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VOL. 14, SEC. C. JULY, 1936 NUMBER 7

THE ABSORPTION OF NUTRIENTS BY TWO VARIETIES OF WHEAT GROWN ON THE BLACK AND GRAY SOILS OF ALBERTA[1]

BY E. K. WOODFORD[2] AND A. G. MCCALLA[3]

Abstract

Chemical analyses carried out at five stages of development of Reward and Red Bobs wheat grown on the black and the gray soils of Alberta showed that differences in soil and variety significantly influenced the composition of the plants.

The weights of dry matter and all nutrients studied were higher for the black-soil plants.

On the basis of percentage dry matter all nutrients, except phosphorus, were higher in the black-soil plants. Reward was higher than Red Bobs in nitrogen when grown on the black soil; and in ash, phosphorus and potassium when grown on the gray soil.

The grain of gray-soil plants was higher in all ash constituents but lower in nitrogen. Varietal differences were more marked in the grain and straw of the mature plants, Reward grain grown on both soils being higher in nitrogen, ash, phosphorus and magnesium.

The total weights, percentages, rates of absorption and ion ratios all indicated that nitrogen and sulphur were limiting the growth of wheat on the gray soil. It is suggested that the proportionately higher absorption of phosphorus from the soil was in compensation for the low availability of nitrogen and sulphur.

The differences in original quality of the wheats grown on the two soils can be largely accounted for by the differences in protein content, and therefore nitrogen supply. Phosphorus absorption, nitrogen, phosphorus and sulphur balance, and the relation of ash to protein, are possibly important in determining the keeping properties of the flour.

Introduction

The black and gray soils of Alberta differ markedly in their chemical composition (23) and in their ability to produce crops of high yield and quality. Larger yields of both grain and forage crops are always obtained on the black soil, and the differences in composition of the crops grown on the two soils cannot be entirely explained by the known differences in soil composition. The quality of the wheat grown on the gray soil is poorer than that of the same varieties grown on the black soil, and the flours milled from most of the varieties deteriorate more rapidly on storage (1). Flour milled from black-soil wheat can be stored for two years without marked deterioration, while flour from similar wheat grown on the gray soil is unfit for commercial bread-making purposes after one year's storage.

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A thorough understanding of the amounts and distribution of the principal nutrients in the wheat plant appeared to be an essential preliminary to the study of the detailed physiological processes which might be responsible for the differences in quality and keeping properties of the flour milled from different varieties of wheat grown on the two soils. The progressive changes in the chemical composition of the developing wheat plant have been followed by many workers, and results from various parts of the world are available. The work of Knowles and Watkin (7) in England is the most comprehensive of recent years. The primary object of these and similar experiments has been, however, to follow the growth of the wheat plant and not to study changes in composition that are caused by soil and varietal differences. Recent work reported from French experimental stations (3, 11, 12, 16) on the chemical composition of different varieties grown on different soils has indicated the magnitude of some of the chemical differences that may be expected. These studies have unfortunately been made at only one or two stages in the plant's development and, as far as the writers are aware, there is no comprehensive work covering this question over the whole life period of the wheat plant. No detailed work has been attempted to relate the chemical development of the wheat plant to the quality and keeping properties of the wheat and flour. The present paper presents the result of such a study in which the absorption of nutrients by two varieties of wheat grown on the black and gray soils was followed over the whole period of development of the plants.

Experimental Material

The material used in this study was grown on fallowed land during the spring and summer of 1934. The experimental plots maintained at Edmonton are typical of the black soil, and those at Fallis of the gray soil areas. Edmonton and Fallis are 50 miles apart, but are characterized by similar climatic conditions. The rainfall is probably slightly higher at Fallis but no meteorological data are available. Since the growing of legumes on the gray soil has been shown to affect the yield and composition of subsequent crops, soil which had never grown legumes was selected for this experiment.

The composition of the two soil types is described by Wyatt and Newton (23) who state that the greatest differences occur in the first foot, where the black soil contains about four times as much nitrogen and more than twice as much phosphorus as the gray soil. There are likewise greater quantities of nitrogen, phosphorus, calcium and magnesium in the second and third foot depths of the black soil than in the corresponding depths of the gray soil.

Two varieties of wheat, Reward and Red Bobs, were grown. Reward was chosen because when grown on the gray soil it had the highest quality of all varieties previously tested, and in addition was the only standard variety commonly grown in this area which produced flour that did not deteriorate rapidly on storage. Red Bobs, when grown on the gray soil, had a marked tendency to produce starchy kernels, and yielded a flour with somewhat poorer original quality and decidedly poorer keeping properties. These two

varieties are, according to grading standards, equal in quality to Marquis and are admitted to all the statutory hard red spring wheat grades.

The experiment was planned to facilitate the statistical analysis of the data. Each variety was replicated eight times on each soil. The varieties were grown side by side in blocks 18 feet long and 12 rows wide. The rows were one foot apart and each variety occupied a sub-block six rows wide. The position of the variety in each block was determined at random. Blocks were adjacent to each other and the whole plot was surrounded by three guard rows.

The plots were sampled five times. Each sample from a sub-block represented the entire length of the block except for a nine inch section at each end which was discarded to remove border effect. The collections were made by a "staircase" method of sampling (Fig. 1). A six inch sample was taken from each row and 27 inches were left between the samples in adjacent rows. The rows in each sub-block and each six inch section in a row were numbered. The starting row and the position of the sample in that row were randomized by the use of Tippet's (18) random sampling numbers. Randomization for all the collections was made at the same time and, if overlapping occurred, the starting point in the row was moved so that two samples were never taken from adjacent six inch sections.

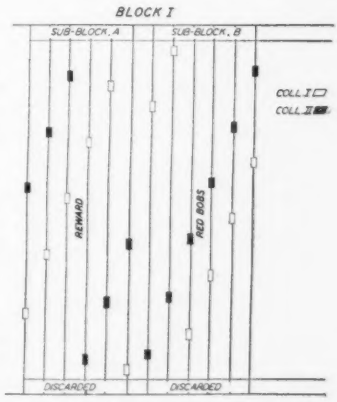

FIG. 1. *Diagram of a section of plot and the method of sampling for the first and second collections.*

Edmonton plants were cut at the ground level and immediately transferred to the laboratory for chemical analyses. Fallis plants were dug from the soil and all the plants from a sub-block transferred to an individual container. In this manner the plants, with roots well covered with soil, were prevented from drying out while they were transported to the laboratory where the tops were cut off just above the ground level immediately before analysis.

Comparable collections were made when the plants were at approximately the same stage of development. Five samplings were made as indicated in Table I. The date of each of the first two collections was governed only by the time from seeding. Collection 3 was made when

TABLE I

Collection	Date of collection		Days from seeding.	
	Edmonton	Fallis	Edmonton	Fallis
1	June 6	June 7	29	29
2	June 26	June 27	48	48
3	July 11	July 12	64	64
4	Aug. 13	Aug. 7	97	90
5	Aug. 31	Aug. 31	115	114

the plants were fully headed, Collection 4 when the grain contained approximately 45% moisture, and Collection 5 at maturity. The heads and straw of Collection 4, and grain and straw of Collection 5, were analyzed separately.

Methods of Analysis

The following determinations were made on each of the eight replicates of each variety, grown at Edmonton and Fallis.

Dry matter. The smaller samples of Collections 1 and 2 were dried directly in a 100° C. air oven. The larger samples of the later collections were rapidly dried in a blast oven at 75° C., then ground in a Wiley mill and further dried at 100° C.

Total nitrogen. Total nitrogen in the vegetative parts was determined by the reduced iron method of Pucher *et al.* (15) in order to include any nitrate nitrogen which was present. The Kjeldahl method, with mercuric oxide as a catalyst, was used to determine nitrogen in the grain, since it has been shown that nitrate nitrogen, if present at all, is negligible.

Non-protein nitrogen. Fresh green material, ground as finely as possible in a food chopper, was extracted with 2.5% trichloracetic acid, and the total nitrogen of this extract determined by the reduced iron method. The procedure was essentially that used by McCalla (9).

Ash. Approximately 2-gm. samples of ground straw or 5-gm. samples of grain were ashed in a muffle furnace at 650° C. for six hours. The ash was taken up in hot dilute hydrochloric acid and the solution diluted to 100 cc. Aliquots of this solution were used in the determination of all the mineral elements studied except sulphur.

Phosphorus. Phosphorus was determined by the colorimetric method described by Truog and Meyer (19) and is reported as phosphorus pentoxide.

Sulphur. Total sulphur was determined only on composite samples of each variety at each place. Approximately 1.5 gm. of the straw, or 0.75 gm. of the grain, was fused in a Parr heat ignition bomb with 12 gm. of sodium peroxide and 0.5 gm. of zinc. The fused mass was dissolved in hot water, made slightly acid, and then filtered. The sulphate was precipitated as barium sulphate and weighed directly. The procedure was an adaptation of the method described by Wolkoff (20).

Potassium. Potassium was precipitated with sodium cobaltic nitrite and the precipitate titrated with $N/20$ potassium permanganate. The details of the procedure are described by Hibbard and Stout (6).

Calcium. Calcium was precipitated as the oxalate and titrated with $N/20$ potassium permanganate.

Magnesium. Magnesium in the filtrate from the calcium determination was precipitated as magnesium ammonium phosphate, dissolved in $N/14$ sulphuric acid, and the excess titrated with $N/14$ sodium hydroxide, methyl red being used as an indicator.

Results

The amounts of the individual nutrients absorbed up to the time of each collection are expressed, first, as total weights; second, as percentages of dry matter; and third, as percentages of total ash. The individual results for each sub-block were too numerous for presentation. The total weights and the mean percentages are presented in graphical form and also in tables with accompanying t values which indicate the significance of the differences due to soil and variety.

The test for significance employed throughout is that of "Student" as described by Fisher (4). The experiment provided eight replicate determinations for each variety at each place. These are taken as providing eight replicate determinations of the differences between Reward and Red Bobs at Edmonton and Fallis. The average of the eight individual differences provided an estimate of the mean difference, and their variance the basis of testing;—(a) whether the mean difference at either place differed significantly from zero (*i.e.*, whether there was any real difference between the varieties) and (b) whether the differences between the varieties were the same at Edmonton and Fallis. In a similar manner, by the use of totals instead of differences, it was determined whether the general level of the various constituents was significantly higher at either Edmonton or Fallis.

These three sets of calculations were made for most of the data. In each of the tables concerned the first column of t values indicates the significance of the difference in the general level at Edmonton and Fallis. The value for the Fallis sample was always subtracted from the value for the Edmonton sample, and in consequence a negative sign means that the general level was higher at Fallis. The second and third columns of t values indicate the significance of the differences between varieties at Edmonton and Fallis, a negative sign meaning that the weight or percentage was higher for Red Bobs than for Reward. The fourth column of t values shows the significance of the difference between the varietal reactions, a high t value indicating that the differences between the varieties were not the same at Edmonton as at Fallis.

Total Weights — Composition of the Whole Plant

The total weights of dry matter, nitrogen, ash and ash constituents are shown in Fig. 2. In the graphs each point, except for sulphur, represents the total of eight replicate samples. The numerical values for these results, together with t values indicating the significance of the differences due to soil and variety, are shown in Table II. Sulphur was determined only on a composite sample from each collection; thus it was impossible to use these data for statistical analysis.

The weights of dry matter and all constituents determined were higher in the Edmonton samples; and the magnitude of this difference, except for potassium and sulphur, increased with the age of the plants. Potassium and sulphur were excreted from the vegetative parts of the plants and the greatest differences in weights occurred before excretion had started. At the point of

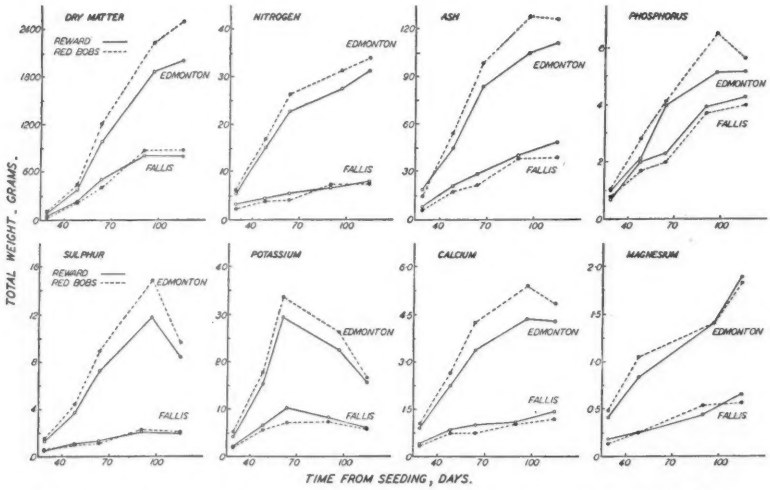

FIG. 2. *Total weights of dry matter, nitrogen, ash and ash constituents absorbed by Reward and Red Bobs from a unit area of soil.*

TABLE II

TOTAL WEIGHTS AND THE SIGNIFICANCE OF DIFFERENCES DUE TO SOIL AND VARIETY

Determination	Collection	Total weight, gm.				*t* values			
		Edmonton		Fallis		Difference between general levels at Edmonton and Fallis	Difference between varieties†		Difference between varietal reactions at Edmonton and Fallis
		Reward	Red Bobs	Reward	Red Bobs		Edmonton	Fallis	
Dry matter	1	100.6	122.5	59.4	53.9	7.11**	−3.12**	0.85	2.81*
	2	380.0	443.4	236.5	233.0	5.57**	−1.60	0.05	1.17
	3	990.4	1223.7	501.2	406.7	25.37**	−4.17**	1.68	4.52**
	4	1863.4	2357.8	814.0	884.3	11.51**	−0.43	0.05	0.34
	5	2011.4	2487.1	827.9	884.6	21.08**	−0.64	−0.46	0.07
Nitrogen	1	5.77	6.74	3.11	2.81	8.21**	−2.30*	0.74	2.16*
	2	15.43	17.07	4.68	4.15	17.35**	−1.13	0.34	1.89
	3	22.98	26.43	5.61	4.27	19.30**	−4.56**	1.68	4.41**
	4	27.53	31.50	6.99	7.54	18.52**	−2.40*	−0.32	1.47
	5	31.04	34.43	8.18	7.79	16.07**	1.76	0.03	1.23
Ash	1	18.81	15.01	8.38	6.90	9.09**	−1.00	1.59	1.83
	2	46.74	54.54	20.81	18.17	12.59**	−2.63*	1.18	2.69*
	3	85.27	99.61	29.47	22.49	13.86**	−5.01**	2.72*	5.47**
	4	106.48	128.63	41.32	40.71	11.50**	1.16	0.95	0.14
	5	112.17	124.04	49.78	40.02	11.32**	−2.28*	0.10	1.69
Phosphorus (P_2O_5)	1	1.02	1.04	0.67	0.72	2.81*	−0.77	−0.46	0.22
	2	2.28	2.71	2.13	1.70	0.93	−1.65	1.21	2.02
	3	3.96	4.16	2.71	2.07	6.53**	−0.54	2.08	1.85
	4	5.19	6.69	3.98	3.71	17.28**	−2.73*	0.44	2.24*
	5	5.31	5.86	4.24	4.04	2.73*	0.94	−0.01	0.67

TABLE II—*Concluded*

TOTAL WEIGHTS AND THE SIGNIFICANCE OF DIFFERENCES DUE TO SOIL AND VARIETY

Determination	Collection	Total weight, gm.				*t* values			
		Edmonton		Fallis		Difference between general levels at Edmonton and Fallis	Difference between varieties†		Difference between varietal reactions at Edmonton and Fallis
		Reward	Red Bobs	Reward	Red Bobs		Edmonton	Fallis	
Potassium	1	4.52	5.23	2.16	2.02	7.12**	−1.29	−0.47	0.58
	2	15.58	17.96	6.86	5.90	11.63**	−1.84	0.75	1.83
	3	29.61	33.90	10.32	7.24	12.24**	−3.23**	1.95	3.66**
	4	22.48	26.34	8.56	7.79	13.43**	−2.03	0.40	1.72
	5	15.80	19.93	6.25	5.70	11.41**	−0.80	0.32	0.79
Calcium	1	0.91	1.09	0.43	0.38	10.57**	−3.21**	0.86	2.88*
	2	2.24	2.66	0.87	0.77	15.92**	−1.88	0.28	1.53
	3	3.37	4.28	1.00	0.77	12.47**	−6.47**	2.04	6.01**
	4	4.37	5.50	1.11	1.10	17.83**	−3.76**	0.00	2.66*
	5	4.30	4.86	1.44	1.24	13.03**	0.85	0.62	0.16
Magnesium	1	0.42	0.48	0.18	0.14	11.30**	−1.62	1.37	2.11
	2	0.84	1.06	0.26	0.26	13.92**	−2.45*	0.28	1.93
	4	1.42	1.42	0.44	0.53	12.45**	0.35	−0.42	0.55
	5	1.84	1.77	0.66	0.56	12.63**	1.92	0.63	0.92

† *A minus sign indicates that the weight for Red Bobs is higher than for Reward.*
* *5% point, t = 2.15.* ** *1% point, t = 2.98.*

the maximum difference the quantities of all constituents except phosphorus, were approximately four times as high in the Edmonton as in the Fallis plants. Phosphorus was exceptional; the differences between Edmonton and Fallis plants being much smaller throughout growth and insignificant in Collection 2.

The differences between varieties indicated that Red Bobs absorbed more nutrients than Reward at Edmonton and less at Fallis, but this difference was significant for less than one-third of the determinations.

Percentage Data

Dry matter as a percentage of green weight. The percentages of dry matter in the fresh samples of the first four collections are shown in the top left hand graph of Fig. 3. The numerical results and *t* values are shown in Table III. Collection 5 is not considered, as it was made after the plants were mature.

Fallis samples were definitely higher in dry matter than corresponding Edmonton samples, and Red Bobs was higher than Reward for both Edmonton and Fallis plants, the latter difference being significant in all but Collections 2 and 3 at Edmonton. Dry matter content is often used as an indication of the stage of maturity of the plant, and composition results are sometimes plotted against the percentage of dry matter instead of the number of days from seeding. In this experiment the percentage of dry matter did suggest the earlier maturity of the Fallis plants, but the higher dry matter content of Red Bobs was not a true indication of maturity since this variety was later than Reward on both soils.

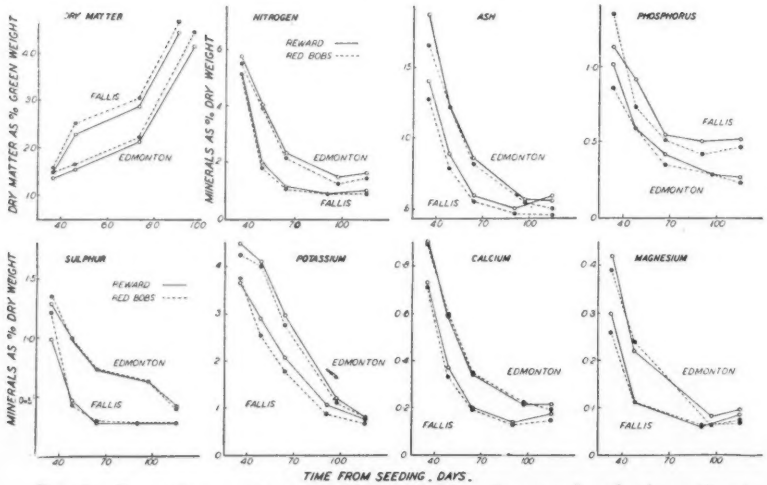

FIG. 3. *Dry matter as a percentage of green weight; nitrogen, ash and ash constituents as a percentage of dry matter in Reward and Red Bobs plants at progressive stages of maturity.*

Nitrogen, ash, and ash constituents, as percentages of dry matter. The average values for nitrogen, ash, and ash constituents expressed as percentages of dry matter are presented graphically in Fig. 3 and numerically, together with *t* values, in Table III.

The percentage results do not directly reflect the sampling error as did the previous total weight results, and therefore, despite the closer proximity of the curves, the differences between them are more significant than in the total weights results of Fig. 2. The general level of all constituents, except phosphorus, was again definitely higher in the Edmonton samples. The proportionately greater concentration of phosphorus in the Fallis plants cannot be attributed to the amount of phosphorus in the gray soil, which is only 60% of that present in the black. Nor can it be due to the greater amount of phosphorus in relation to the other elements, because in relation to calcium and magnesium, phosphorus is less in the gray than in the black soil.

The higher level of constituents in Reward was significant for more than 50% of the determinations, the difference between the varieties being in general greater at Fallis for all constituents except nitrogen.

Despite the general similarity of all the curves, the more rapid drop in nitrogen and sulphur of the Fallis plants indicates that these elements were more limiting in the gray soil. During the first month the Fallis plants absorbed approximately 37% of their total nitrogen and 28% of their total sulphur, while the corresponding absorption for Edmonton plants was 19% and 12% respectively. Although phosphorus was definitely higher in the Fallis plants, the percentage of the total uptake of this element absorbed during the first month of growth was 17% at Fallis compared with 16% at Edmonton, indicating that phosphorus was equally available at Fallis and Edmonton.

TABLE III

PERCENTAGE DATA AND THE SIGNIFICANCE OF DIFFERENCES DUE TO SOIL AND VARIETY

Determination	Collection	Mean of 8 replicate samples				*t* values			
		Edmonton		Fallis		Difference between general levels† at Edmonton and Fallis	Difference between varieties††		Difference between varietal reactions at Edmonton and Fallis
		Reward	Red Bobs	Reward	Red Bobs		Edmonton	Fallis	
Dry matter, % green weight	1	13.4	14.6	14.8	15.5	−2.94*	−6.89**	−4.05**	2.01
	2	15.3	16.3	22.8	25.1	−9.13**	−1.35	−3.44**	1.47
	3	21.2	22.2	28.8	30.4	−4.33**	−1.82	−3.13**	0.92
	4	41.8	44.8	44.3	46.7	−2.87*	−3.43**	−2.75*	0.46
Nitrogen, % dry matter	1	5.74	5.50	5.24	5.22	6.55**	3.80**	−0.05	2.90*
	2	4.06	3.85	1.98	1.78	9.36**	2.91*	3.64**	0.52
	3	2.32	2.16	1.12	1.05	12.36**	3.15**	1.29	1.32
	4	1.48	1.33	0.86	0.85	24.97**	6.86**	0.39	4.61**
	5	1.54	1.39	0.99	0.88	17.35**	8.63**	3.80**	3.42**
Ash, % dry matter	1	18.70	16.69	14.14	12.78	12.16**	5.03**	3.70**	0.94
	2	12.28	12.30	8.84	7.80	13.45**	0.38	3.71**	2.35*
	3	8.61	8.14	5.88	5.53	9.34**	2.58*	1.93	0.45
	4	5.70	5.45	5.14	4.60	3.77**	1.25	2.68*	1.01
	5	5.50	4.93	5.88	4.51	0.12	2.03	3.99**	1.39
Phosphorus (P_2O_5), % dry matter	1	1.01	0.85	1.13	1.33	− 4.57**	4.59**	−5.30**	6.99**
	2	0.60	0.61	0.90	0.73	− 2.08	−0.24	3.39**	2.42*
	3	0.40	0.34	0.54	0.51	− 4.80**	4.55**	1.95	1.84
	4	0.28	0.28	0.50	0.42	−11.01**	0.06	4.92**	3.44**
	5	0.26	0.23	0.51	0.46	− 8.89**	1.75	2.91*	0.81
Potassium, % dry matter	1	4.49	4.26	3.64	3.74	3.88**	1.45	0.16	1.14
	2	4.10	4.05	2.90	2.53	12.72**	0.27	2.73*	1.74
	3	2.99	2.77	2.06	1.78	11.51**	3.08**	3.51**	0.30
	4	1.21	1.12	1.06	0.88	5.54**	2.64*	5.38**	1.94
	5	0.78	0.78	0.75	0.65	2.65*	−0.05	3.86**	2.76*
Calcium, % dry matter	1	0.90	0.89	0.73	0.71	5.79**	0.60	1.14	0.38
	2	0.59	0.60	0.37	0.33	9.48**	0.50	1.85	1.66
	3	0.34	0.35	0.20	0.19	17.95**	−0.93	1.24	1.53
	4	0.22	0.28	0.13	0.13	17.68**	−0.98	0.98	1.39
	5	0.22	0.19	0.17	0.14	8.84**	2.56*	3.31**	0.53
Magnesium, % dry matter	1	0.42	0.39	0.30	0.26	9.71**	1.25	1.74	0.35
	2	0.22	0.24	0.11	0.11	18.22**	−1.39	0.56	1.37
	4	0.080	0.060	0.058	0.060	3.46**	4.81**	−0.44	3.71**
	5	0.093	0.071	0.081	0.063	2.64*	2.89*	2.64*	0.17

† *A minus sign indicates that the percentage is higher in the Fallis plants.*
†† *A minus sign indicates that the percentage for Red Bobs is higher than for Reward.*
* *5% point, t = 2.15.*
** *1% point, t = 2.98.*

Various workers (8) have shown that there is a reciprocal relationship between the amounts of nitrogen and phosphorus absorbed by plants and the percentages of these elements occurring within the plant. Low nitrogen supply, together with high available phosphorus, may then be responsible for the definitely higher concentrations of phosphorus in the Fallis plants.

Sulphur is also important in this connection. Marsh (10) has shown that a reciprocal relationship existed between sulphur and phosphorus in the tomato plant. The high concentration of phosphorus in the Fallis samples may then be due in part to the effect of low nitrogen, and in part to the effect of low sulphur.

Ash constituents as a percentage of total ash. The average values for phosphorus, sulphur, potassium and calcium expressed as percentages of the total ash are presented graphically in Fig. 3 and numerically, together with t values, in Table IV.

TABLE IV

MINERALS AS A PERCENTAGE OF ASH AND THE SIGNIFICANCE OF DIFFERENCES DUE TO SOIL AND VARIETY

Determination	Collection	Mean of 8 replicate samples				t values			
		Edmonton		Fallis		Difference between general levels† at Edmonton and Fallis	Difference between varieties††		Difference between varietal reactions at Edmonton and Fallis
		Reward	Red Bobs	Reward	Red Bobs		Edmonton	Fallis	
Phosphorus (P_2O_5)	1	5.41	5.08	7.98	10.40	−13.15**	1.14	−8.58**	6.87**
	2	4.87	4.93	9.70	9.26	−18.90**	−0.16	1.05	0.86
	3	4.68	4.12	9.17	9.08	−16.87**	2.68*	0.40	1.62
	4	4.98	5.15	9.63	9.20	−15.25**	−0.52	1.30	1.29
	5	4.83	4.73	8.60	10.27	− 7.45**	0.33	−5.39**	4.05**
Potassium	1	24.2	25.5	25.9	29.2	−2.54*	−0.11	−2.78*	1.89
	2	33.5	33.1	32.8	32.4	0.72	0.32	0.36	0.03
	3	34.8	33.9	34.4	32.3	1.38	0.98	2.50*	1.07
	4	21.2	20.5	20.7	19.2	0.72	4.25**	6.91**	1.88
	5	14.4	16.0	12.8	14.5	1.14	−2.43*	−2.57*	0.10
Calcium	1	4.83	5.32	5.33	5.54	−1.59	−2.07	−0.15	1.36
	2	4.76	4.72	4.11	4.27	6.01**	−0.57	−1.17	0.42
	3	3.91	4.32	3.22	3.38	2.61*	−6.80**	−2.63*	2.95*
	4	3.84	4.21	2.25	2.72	10.66**	−2.40*	−0.39	1.42
	5	3.89	3.96	2.94	3.20	7.23**	−0.52	−1.86	0.95

† *A minus sign indicates that the percentage is higher in the Fallis plants.*

†† *A minus sign indicates that the percentage for Red Bobs is higher than for Reward.*

* *5% point, $t = 2.15$.*

** *1% point, $t = 2.98$.*

Unlike the previous results the graphs for the individual constituents are not similar. Phosphorus remained fairly constant in the samples from each soil, but was almost twice as high in the Fallis samples. The outstanding difference between the two sets of sulphur curves is again an indication that this element is definitely limiting in the gray soils. Potassium, however, was almost identical for both varieties and soils. While the lower calcium content in the Fallis plants may indicate a lower supply of this element in the gray soil, the slight increase in the last Fallis collection is significant, and is shown in another section of this paper to be reflected in the composition of the grain.

There were no consistent significant differences between the varieties, although there were indications that Red Bobs was higher in sulphur and calcium on both soils. Likewise there were no consistent significant differences between the reactions of the varieties to the two soils.

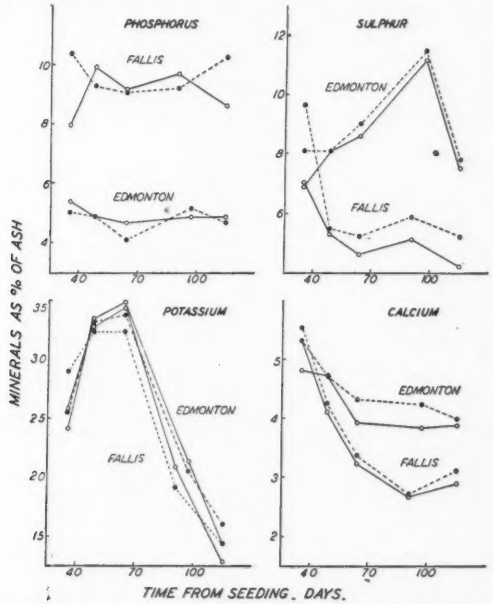

FIG. 4. *Ash constituents as a percentage of total ash in Reward and Red Bobs plants at progressive stages of maturity.*

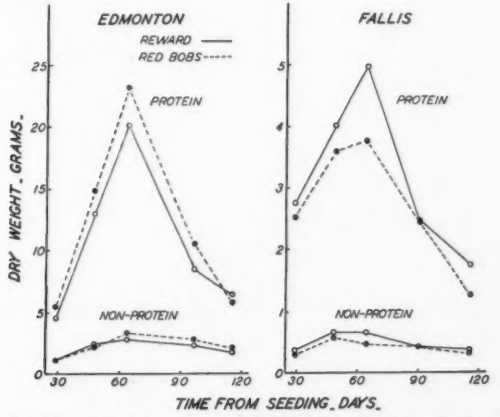

FIG. 5. *Total weights of protein and non-protein nitrogen in the vegetative parts of Reward and Red Bobs from a unit area of soil.*

Nitrogen Fractions

Total nitrogen was separated into protein and organic non-protein fractions. Nitrate nitrogen was also determined in Collection 1, but this fraction was less than 1% of the total nitrogen and is not considered.

The total weights of protein and non-protein nitrogen in the vegetative parts of all collections are represented graphically in Fig. 5. Although the total weights in Edmonton samples were nearly five times as high as those of the Fallis samples, the relative changes in the amounts of total and non-protein nitrogen were in general the same.

Non-protein nitrogen as a percentage of the total nitrogen in the vegetative parts is shown in Fig. 6. The curves overlap only at the time of the second and third collections. As soon as translocation of nitrogen to the grain began there was a larger proportion of the non-protein nitrogen in the vegetative parts of the Edmonton plants due, if the results of comparable water culture experiments are applicable (9), to a proportionally greater nitrogen absorption after heading. This is further substantiated by the fact that the Edmonton plants absorbed on the average 8 gm. of nitrogen after Collection 3, while the Fallis plants absorbed only 3 gm., and is additional evidence that nitrogen was limiting development under Fallis conditions.

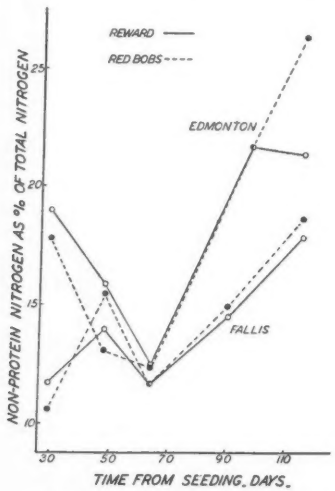

FIG. 6. *Non-protein nitrogen as a percentage of total nitrogen in the vegetative parts.*

Equivalent Uptake of Anions and Cations

The results so far discussed lead to the general conclusion that phosphorus is relatively more available than nitrogen and sulphur in the Fallis soil. The importance of this relationship in altering the composition of the plant is shown in Fig. 7, where the ratios nitrogen : phosphorus and sulphur : phosphorus in the plants at the various collections are plotted. The ratios are calculated from the equivalent weights of the elements, and the graphs show that both of these ratios were definitely higher at all times in the Edmonton plants. This was to be expected if nitrogen and sulphur were limiting at Fallis and phosphorus was readily available.

It has already been suggested that the Fallis plants absorbed large amounts of phosphorus to compensate for the low availability of nitrogen and sulphur. If this reciprocal relation between the anions of nitrogen, sulphur and phosphorus did in some way tend to control the total amount of anions absorbed, then there should be a fairly constant relationship between the principal anions and cations absorbed from each soil. This anion : cation ratio was

calculated in terms of equivalents for the two varieties for all collections and is also shown in Fig. 7. The similarity of the curves for both varieties and soils substantiates the hypothesis that there is a controlling influence which

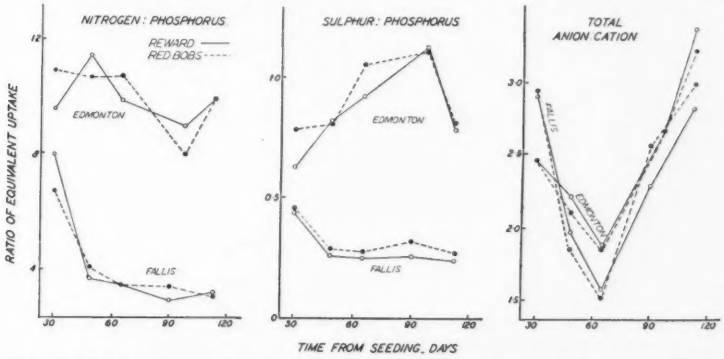

FIG. 7. *Ratios of the equivalent uptake of nitrogen and phosphorus, sulphur and phosphorus, and total anions : cations in Reward and Red Bobs at progressive stages of maturity.*

regulates the proportion of anions to cations absorbed, despite the limiting supply of two of the anions. The high phosphorus content of the Fallis plants was then probably not due to the greater availability of phosphorus in the Fallis soil, but rather to the influences controlling the anion : cation balance.

Percentage Rate of Uptake of Plant Nutrients

Besides the differences in total amounts of nutrients absorbed from the two soils there was a marked difference in the rates of nutrient uptake between collections. It has already been noted that the amounts of nitrogen and sulphur absorbed during the first month of growth were proportionately much greater at Fallis. To illustrate this difference in the rate of uptake the average percentage absorption between collections was calculated. The weight of an element absorbed between successive collections was divided by the number of days between the collections, and this value expressed as a percentage of the total amount of the element absorbed. These percentages for nitrogen, phosphorus, sulphur and potassium, in Reward grown on both soils, are plotted against the number of days from seeding in Fig. 8. The data of this experiment are somewhat inadequate for this type of calculation, but the general shape of the curves clearly demonstrates the point in question.

The increasing rate of nitrogen absorption by the Edmonton plants until Collection 3 indicates that as the plants increased in size their increased nitrogen needs were supplied. The fall in the rate of nitrogen absorption by the Fallis plants means, on the other hand, that there was a limitation of this element at or before the time of the first collection. The somewhat similar relationship between the percentage rates of sulphur absorption can be explained in the same way. The similarity of the phosphorus and potassium curves is evidence that these elements were not seriously limiting in either soil.

It appears that, under the more favorable Edmonton soil conditions, where the supply of these four nutrients can be assumed to be sufficient for excellent growth, the percentage rate of absorption of them by the plant increased until about the time of heading. At Fallis this maximum for the absorption of nitrogen and sulphur was reached at about the time of the first collection, which can only be attributed to the low availability of these two elements in the gray soil.

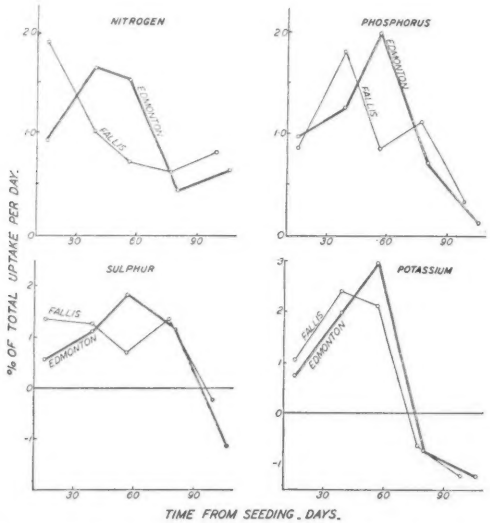

FIG. 8. *The percentage rate of uptake of nutrients by Reward at progressive stages of maturity.*

The foregoing results on the composition of the whole plant have shown the very definite effect of the soil, not only on yield, but also on the composition of the plant. The black soil produces a higher yield of wheat which is richer in all nutrients except phosphorus. The difference in phosphorus is exceptional because as a percentage of dry matter or ash it is significantly higher in the Fallis plants at all stages of maturity, despite the fact that soil analyses show that the total phosphorus in the gray soil is only 60% of that in the black.

Fertilizer experiments (13, 23) have shown that nitrogen and sulphur are limiting in the Fallis soil and the results presented in this paper in every case substantiate these findings. The shape of the total-weight curves, the ratios of equivalent uptake, the percentage rate of uptake, and the low percentage of nitrogen in the Fallis samples, all indicate that nitrogen and sulphur were limiting growth at Fallis early in the development of the plant.

In addition, it was shown that the anion : cation ratio was approximately the same for plants grown on both soils. It is suggested, therefore, that there is a controlling influence which maintains a fairly constant anion : cation ratio

under different nutritional conditions and that under gray soil conditions, where two of the important anions are limiting, the anion : cation ratio is maintained at approximately the usual level by the greater absorption of phosphorus.

The effect of variety in determining the composition of the wheat plant has generally been observed in its effect on the composition of the mature grain or straw. No systematic study of the effect of variety in controlling the composition of the whole plant at progressive stages of maturity has come to the attention of the authors. This study shows that for certain elements expressed as a percentage of dry matter the composition of the two varieties was significantly different at practically all stages of development. Reward was significantly higher than Red Bobs in nitrogen at all collections on the black soil and in total ash, phosphorus and potassium for four collections on the gray soil.

Composition of Straw and Grain at Maturity

Total Weights

The total weights of dry matter, nitrogen, ash and ash constituents in grain and straw of mature plants are shown as bar diagrams in Fig. 9, and numerically together with t values, in Table V.

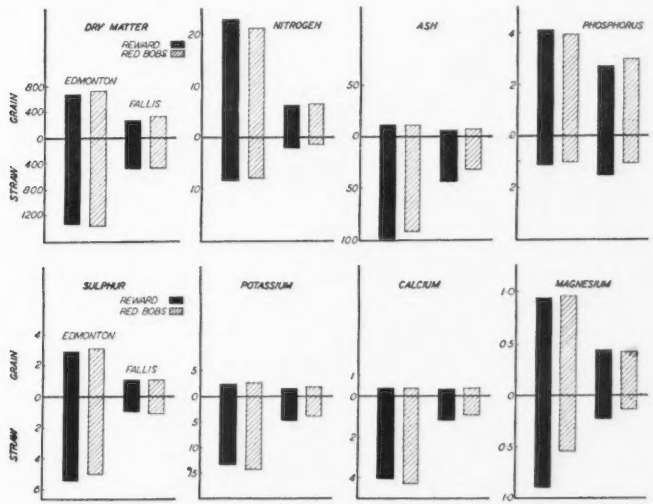

FIG. 9. *Total weight in grams of dry matter, nitrogen, ash, and ash constituents, in grain and straw of the final collection.*

The weights of all constituents were significantly higher in the Edmonton samples except for phosphorus in the straw and calcium in the grain where there were no definite differences between the samples from the two soils. Differences between varieties were significant in only two instances.

TABLE V

TOTAL WEIGHTS IN STRAW AND GRAIN OF MATURE PLANTS AND THE SIGNIFICANCE OF DIFFERENCES DUE TO SOIL AND VARIETY

Determination	Part of plant	Total weight, gm.				*t* values			
		Edmonton		Fallis		Difference between general levels† at Edmonton and Fallis	Difference between varieties††		Difference between varietal reaction at Edmonton and Fallis
		Reward	Red Bobs	Reward	Red Bobs		Edmonton	Fallis	
Dry matter	Grain	675.1	728.6	263.6	324.3	10.24**	−1.35	−1.55	0.14
	Straw	1336.3	1362.0	564.0	560.0	19.55**	−0.30	0.04	0.24
Nitrogen	Grain	22.75	21.06	6.09	6.19	16.00**	1.74	−0.02	1.24
	Straw	8.29	8.04	2.09	1.60	10.98**	0.69	−0.55	0.10
Ash	Grain	11.68	11.95	5.48	6.29	10.80**	−0.02	−0.27	0.20
	Straw	100.49	92.90	44.30	33.73	10.69**	1.41	1.46	0.00
Phosphorus (P_2O_5)	Grain	4.15	3.89	2.66	2.95	4.00**	0.06	−0.97	1.10
	Straw	1.16	1.06	1.58	1.09	−1.28	0.16	3.10**	2.08
Potassium	Grain	2.30	2.55	1.40	1.72	4.61**	−1.52	−1.71	0.14
	Straw	13.50	14.30	4.85	3.98	22.42**	−0.67	0.83	1.05
Calcium	Grain	0.28	0.29	0.26	0.29	0.32	0.03	1.13	0.77
	Straw	4.01	4.60	1.19	0.95	13.75**	0.92	0.84	0.06
Magnesium	Grain	0.95	0.95	0.44	0.42	9.37**	−0.23	0.19	0.29
	Straw	0.90	0.55	0.22	0.14	9.70**	2.52*	0.77	1.26

† *A minus sign means the weight is higher for Fallis plants.*

†† *A minus sign means the weight is higher in Red Bobs than Reward.*

* *5% point, t = 2.15.*

** *1% point, t = 2.98.*

The percentages of nutrients translocated to the grain are shown in Table VI. At Fallis, Red Bobs produced more grain in comparison with total plant than Reward, and there was a greater translocation of all nutrients except sulphur. At Edmonton this difference between the varieties was not so marked, and the percentage of nutrients translocated to the grain was approximately the same for both varieties.

TABLE VI

THE WEIGHT OF DRY MATTER, ASH AND NUTRIENTS IN THE GRAIN AT MATURITY EXPRESSED AS A PERCENTAGE OF THE TOTAL IN THE WHOLE PLANT

Collection		Dry matter	Nitrogen	Ash	Phosphorus	Sulphur	Potassium	Calcium	Magnesium
Edmonton	Reward	33.6	73.2	10.4	78.2	34.6	14.6	6.6	51.4
	Red Bobs	34.8	72.4	11.4	78.5	32.3	15.1	6.7	63.6
Fallis	Reward	31.8	74.4	11.0	62.7	58.9	22.4	17.9	65.9
	Red Bobs	36.7	79.5	15.7	73.0	53.7	30.2	23.1	75.0

The total weights of dry matter and nutrients in 1000 kernels are presented in Table VII. The weight of any element occurring in the grain is dependent upon the total absorption of that element, the percentage of it translocated to

TABLE VII

WEIGHT OF DRY MATTER AND NUTRIENTS PER 1000 KERNELS, AND THEIR RELATION TO NITROGEN

Sample		Dry matter	Nitrogen	Ash	Phosphorus	Sulphur	Potassium	Calcium	Magnesium
Wt. of 1000 kernels									
Edmonton	Reward	26.3	1.007	0.357	0.184	0.129	0.102	0.0126	0.0419
	Red Bobs	26.7	0.876	0.456	0.162	0.130	0.106	0.0121	0.0397
Fallis	Reward	24.1	0.636	0.570	0.277	0.123	0.145	0.0269	0.0452
	Red Bobs	26.1	0.565	0.574	0.269	0.106	0.157	0.0260	0.0385
Nitrogen basis 100									
Edmonton	Reward		100	35.5	18.3	12.8	10.1	1.3	4.2
	Red Bobs		100	52.1	48.5	14.8	12.1	1.4	4.5
Fallis	Reward		100	89.6	43.6	19.3	22.8	4.2	7.1
	Red Bobs		100	101.5	47.6	18.8	27.8	4.6	6.8

the grain, and the yield of grain. Nitrogen was higher in the Edmonton grain because of the proportionately greater absorption of this element by the Edmonton plants, and for similar reasons, phosphorus was higher in the Fallis grain. Sulphur absorption was also higher at Edmonton, but because of the greater percentage translocation of this element to the grain of the Fallis plants the composition of the grain was not greatly altered. The percentage of all cations was higher in the Fallis grain because there was a greater proportion of these elements translocated to the grain.

The outstanding differences in the composition of the various grain samples are shown in the lower section of Table VII, where the weight of each of the constituents is compared with nitrogen expressed as 100. The higher proportion of ash constituents in the Fallis grain is very definite and may be of significance in influencing the quality of the grain (2, 5). The differences between varieties were not so great, but Red Bobs was higher in the proportion of total ash and slightly higher in the proportion of potassium and phosphorus.

Percentage Data

Nitrogen, ash, and ash constituents, as a percentage of dry matter. Nitrogen, ash, and ash constituents of the final collection expressed as a percentage of dry matter are shown in Table VIII, together with accompanying t values.

The higher percentages of nitrogen and all ash constituents, except phosphorus, in the straw of Edmonton plants were in accord with the previous results on the composition of the whole plant. This condition was, however, reversed in the grain where all the constituents, except nitrogen, were definitely higher in the Fallis samples. The exceptionally large amounts of calcium and phosphorus were undoubtedly due to the greater translocation of the former (Table VI) and greater absorption of the latter at Fallis.

TABLE VIII

PERCENTAGE DATA FOR STRAW AND GRAIN OF THE MATURE PLANTS, AND THE SIGNIFICANCE OF DIFFERENCES DUE TO SOIL AND VARIETY

Determination	Part of plant	Mean of 8 replicate samples				*t* values			
		Edmonton		Fallis		Difference between general levels† at Edmonton and Fallis	Difference between varieties††		Difference between varietal reaction at Edmonton and Fallis
		Reward	Red Bobs	Reward	Red Bobs		Edmonton	Fallis	
Percentage of dry matter									
Nitrogen	Grain	3.37	2.89	2.31	1.91	22.37**	50.30**	43.65**	6.12**
	Straw	0.62	0.59	0.37	0.32	5.92**	9.82**	9.88**	1.13
Ash	Grain	1.70	1.57	2.08	1.94	− 6.12**	7.51**	5.02**	1.77
	Straw	7.52	6.82	7.85	6.02	1.01	4.95**	10.42**	3.87**
Phosphorus	Grain	0.61	0.53	1.01	0.91	−18.90**	3.47**	5.09**	1.38
	Straw	0.087	0.078	0.280	0.194	− 8.80**	0.51	4.95**	3.14**
Sulphur	Grain	0.43	0.43	0.45	0.36	—	—	—	—
	Straw	0.41	0.37	0.15	0.18	—	—	—	—
Potassium	Grain	0.34	0.35	0.53	0.53	−15.85**	−0.53	0.19	0.60
	Straw	1.01	1.05	0.86	0.71	5.17**	−0.06	3.84**	2.76*
Calcium	Grain	0.042	0.040	0.098	0.088	− 4.72**	1.80	1.79	0.65
	Straw	0.30	0.28	0.21	0.17	12.07**	2.31*	3.08**	0.55
Magnesium	Grain	0.140	0.131	0.165	0.130	− 2.44*	2.76**	10.45**	5.44**
	Straw	0.067	0.040	0.040	0.025	4.62**	2.95**	1.55	0.99
Percentage of ash									
Phosphorus	Grain	35.5	32.9	48.5	46.8	−10.89**	1.73	1.34	0.65
	Straw	1.16	1.17	3.60	3.21	−14.72**	−0.34	2.24*	1.82
Potassium	Grain	20.0	22.5	25.6	26.8	− 3.65**	−1.53	−0.76	0.55
	Straw	13.6	15.1	11.0	11.9	3.31**	−2.52*	−1.36	0.82
Calcium	Grain	2.48	2.43	4.69	4.57	−10.12**	0.29	0.61	0.22
	Straw	4.02	4.13	2.71	2.91	6.63**	−0.70	−1.28	0.42

† *Minus sign indicates a higher percentage in Fallis plants.*
†† *Minus sign indicates a higher percentage in Red Bobs than Reward.*
* *5% point, t = 2.15.*
** *1% point, t = 2.98.*

Varietal differences were definite, the percentage of all constituents, except potassium at Edmonton, being higher in Reward despite the greater translocation of nutrients to the grain by Red Bobs. The varietal differences were on the whole more definite in the composition of the straw.

The differences between the varietal reactions at Edmonton and Fallis showed that the percentage of nutrients in Reward was higher on both soils, but that this difference between the varieties was often greater at Fallis. Nitrogen in the grain was exceptional, the difference between the two varieties being significantly higher at Edmonton.

Ash constituents as a percentage of total ash. Phosphorus, potassium and calcium expressed as percentages of the total ash in the final collection are also shown in Table VIII, together with their accompanying t values.

These results show the same relation between soils as did the previous results expressed on a dry matter basis. The differences between varieties were, however, no longer significant, indicating that the composition of the ash was not altered as much as the composition of the dry matter.

These results show that the soil very definitely influenced the composition of the grain and straw of the final collection. The percentage of all ash constituents, except phosphorus, translocated to the grain was so much greater at Fallis that the composition of the grain became richer and the composition of the straw poorer than at Edmonton. The higher proportion of the calcium translocated to the grain in the Fallis plants resulted in a percentage of calcium twice as high as in the Edmonton grain. Similarly there was a much greater translocation of sulphur in the Fallis plants, but owing to the limiting supply of this element the greater translocation only brought the percentage of sulphur in the Fallis grain to approximately the same level as that in the Edmonton grain. It did, however, markedly lower the sulphur content of the Fallis straw. Translocation to the grain tends to be so regulated that under very diverse nutritional conditions the composition of the grain will be much more nearly uniform than that of the straw, and these results show that the effect of soil or variety in altering the composition of the plant was much greater in the composition of the straw than in the composition of the grain or the whole plant.

The ratio of grain to straw and the proportion of nutrients translocated to the grain determine in a large measure the composition of the grain and influence the quality of the flour into which it can be milled. Red Bobs produced on both soils a greater proportion of grain to straw than Reward, and the difference between the varieties was greater at Fallis. This greater proportionate production of grain by Red Bobs when grown under the less favorable gray soil conditions was accompanied by a greater proportionate translocation of nutrients to the grain, but despite this, the grain of Red Bobs was lower in most constituents and poorer in quality than that of Reward.

Quality of Grain

The quality of the wheats grown in this experiment was determined by the experimental baking procedure using the bromate formula. During 1932 and 1933 Reward and Red Bobs were grown under conditions similar to those of this experiment, and in Table IX the quality of the wheats for the three years is compared by means of protein and loaf volume results.

The differences in loaf volume results show, as has previously been indicated, that Reward was in general higher in quality than Red Bobs at both Edmonton and Fallis. This original quality of the wheats is closely related to protein content, and both loaf volume and protein results show that this relationship between the varieties was, with one exception, the same for all three years.

TABLE IX

Sample		Protein, %			Loaf volume, cc.		
		1932	1933	1934	1932	1933	1934
Edmonton	Reward	16.5	16.8	16.6	708	795	668
	Red Bobs	14.2	14.7	14.2	632	694	545
Fallis	Reward	13.6	12.6	11.4	662	627	515
	Red Bobs	10.3	10.6	9.4	588	632	464

There was, however, a considerable fluctuation in the results from year to year. The 1934 loaf volume results are particularly low in comparison with the 1932 and 1933 results. This can be attributed in part to the climatic conditions of the year, since nearly all wheat grown in central and northern Alberta was of comparatively low quality in 1934, and in part to the effect of a frost on August 22. The frost occurred when the percentage of dry matter in the grain was approximately 58%. Newton and McCalla (14) have concluded that when wheat contains 58% dry matter it has reached a critical stage of development, after which little or no translocation of nutrients takes place. Thus it is assumed that in this experiment translocation to the grain was not affected, although the baking quality of the flour, as indicated by loaf volume results, may have been slightly lowered.

Discussion

The results of the chemical analyses of the developing plants have been expressed in a number of different ways in order that as many differences as possible between varietal and soil reactions might be emphasized. It is unfortunate that the individual total-weight results were not more accurate but the stands of wheat, particularly at Fallis, were not uniform and the samples from each sub-block often varied greatly even though the total weights per collection, representing 48 six-inch sections, undoubtedly were fairly accurate. It was impossible to use bigger samples because of the difficulty in handling in the laboratory but, had the large sampling error been anticipated, the method of sampling might have been improved by collecting a fixed number of plants and then determining the comparative yields of the varieties on the two soils by counting the number of plants per unit length of row.

The importance of soil and variety in affecting the composition of the plant, the quality of the wheat and the keeping properties of the flour has been amply emphasized. Nitrogen absorption and the percentage of protein in the grain are undoubtedly among the main factors in determining the original quality of the grain, but the varietal differences in the deterioration of the flour must be due to other causes, and more detailed studies are necessary before these differences can be definitely related to the differences in absorption and translocation of nutrients already noted.

Wood (21) as early as 1907 suggested that the development of the plant might affect the quality of the proteins produced in the grain. He believed that acids and salts in the cell sap during endosperm formation impress upon the gluten the physical properties that decide the character of the flour obtained from that wheat. Gericke (5) extended this idea, and suggested that the plant sap, even before endosperm formation, might influence directly the quality of the proteins. He has, with other workers (2), stressed the particular importance of minerals in this regard. It is interesting to note that in this particular experiment the ratio of minerals to protein was higher at Fallis and higher in Red Bobs than in Reward, but the relation between nutrition and keeping properties of flour is a different problem from that under discussion by Wood and Gericke.

In the Fallis plants the nitrogen-phosphorus-sulphur balance is very different from that in the Edmonton plants. Since these elements are important constituents of the proteins it is possible that the composition of the Fallis grain was altered in some way which affected quality. If this is the cause, the reason for the much more rapid deterioration of Red Bobs flour is not clear.

The importance of lipoidal substances in affecting the physical properties of the wheat proteins is also known (22). Storage experiments (17) with flour from Edmonton and Fallis wheat have shown that there are greater changes in the lipoidal substances in the Fallis flour, and it seems certain that these changes are related to the deterioration of the baking quality of the flour. Phosphorus is an important constituent of these lipoidal substances, and the greater uptake of phosphorus by plants grown at Fallis may in some way alter the metabolism of these plants so that the particular lipoidal substances formed are more susceptible to alteration than similar substances formed in the grain grown at Edmonton.

The studies reported in this paper have not necessarily shown all of the important differences which may exist in the mineral nutrition of wheat grown on the black and gray soils. The importance of other elements needed in smaller amounts has been increasingly recognized in plant nutrition studies, and it seems equally possible that some of these might influence the keeping properties of flour. Studies on such elements have not been undertaken, but offer possibilities.

At the present time studies on the details of nitrogen, phosphorus and sulphur metabolism appear to be the most promising. Such studies are at present under way.

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FROST-HARDENING STUDIES WITH LIVING CELLS

I. OSMOTIC AND BOUND WATER CHANGES IN RELATION TO FROST RESISTANCE AND THE SEASONAL CYCLE[1]

By J. Levitt[2] and G. W. Scarth[3]

Abstract

The osmotic pressure and non-solvent space of the cells of various types of plant were estimated by the plasmolytic method and related to frost resistance and the seasonal cycle.

Osmotic pressure always rises with hardening and falls with dehardening, and it generally reaches higher values or begins to rise earlier in the hardier species and varieties.

The effect of osmotic pressure in reducing the amount of ice formation is enhanced in woody plants by the condition that only about half the cell volume is occupied by the osmotically active solution. The remainder, *i.e.*, the non-solvent space, is shown to consist partly of bound water and must therefore represent hydrophilic colloid. This occupies an even larger proportion of the sap vacuole than of the protoplasm, and it increases notably with hardening. This change, besides reducing intercellular ice, is regarded as protecting the most vulnerable part of the cell, *viz.*, the vacuole, from being frozen at very low temperatures.

Introduction

The process of hardening against frost produces in extreme cases a remarkable increase in power of resistance. Cells which were sensitive to a few degrees of frost may become capable of enduring $-40°$ or even $-80°$ C. without injury. To account for this, some drastic change in the condition or physiology of the tissues might be anticipated. Yet, while various physical and chemical changes have been noted, the mechanism of frost resistance is still largely a mystery. Perhaps attention has been devoted too exclusively to the properties of tissue extracts and too little to living cells. It is on the latter that we have concentrated, in a series of studies beginning with the present paper. The chief advantages of cell study are these, (i) it is possible to detect local differences in physico-chemical properties not only in individual cells but in different parts of a cell; (ii) it deals with the protoplasmic and other colloids in their natural state—not profoundly altered by death; (iii) it allows investigation of functions and properties which are peculiar to life.

Another distinctive feature of our plan is to use in part cells which are capable of extreme cold resistance, and in which the hardening changes must therefore be greatest. For this reason, we have studied largely the twigs of woody plants, which have no protection in winter.

Review of Literature

It is now a generally established principle that during the hardening period an increase in the osmotic pressure of the cell sap occurs. This is usually due to a hydrolysis of starch to sugar but there may be other causes. Elabora-

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tion of glucosides, such as anthocyanins, has been correlated with cold resistance (40) and, similarly, conversion of disaccharides to monosaccharides. Besides the actual change in the quantity of solute, an alteration in the amount of solvent may be just as effective in modifying the osmotic pressure of the cell sap. Thus, if part of the water is colloidally adsorbed ("bound water"), a change in the water-holding power of the cell, and therefore of its "osmotic pressure", will result. The latter question will be considered in the second half of this paper.

In by far the majority of the papers which have any bearing on osmotic pressure changes, the starch⇌sugar equilibrium is involved.

Russow (37) asserted in 1884 that starch disappears during November and reappears in March. Müller-Thurgau (32) showed in 1886 that exposure of plants (*e.g.*, potatoes) to low temperatures causes a hydrolysis of starch to sugars. However, at this time the consensus of opinion appeared to be that the phenomenon was pathological. It remained for Lidforss (25, 26) to extend and expand their observations. In an exhaustive survey of 130 phanerogams, he was able to show that the winter season causes a complete disappearance of starch from the leaves of evergreens and a corresponding increase in soluble sugars. Several conversions may occur during the winter with changes in temperature. Miyake (30) subdivides Japan into three regions according to the severity of the winter. In the coldest of these, almost all the starch disappears, but in the other two, greater or less amounts are still to be found in overwintering leaves. Specific differences are also well-known (11, 20), as well as variation with the tissue and the character of the cells (41). Even in the severe Canadian winter the pith rays and wood parenchyma of some trees may be packed with starch.

Lidforss (26) further showed that the evergreens are resistant to cold only when their tissues possess these high quantities of sugars. As soon as starch appears in their tissues, frost resistance is lowered. It is but a short step from here to attempt a distinction in the relative winter hardiness of plants by estimating the quantities of sugars occurring in them. Numerous efforts have been made to distinguish in this way between hardy and tender varieties of a species. The group of plants which has served most as experimental material is the winter cereals.

Gassner and Grimme (15) determined the sugar content, and Newton (33) and Martin (28) the depression of the freezing point of the sap of wheat varieties, and found the values to be proportional to their hardiness. Åkerman (1) in particular exhaustively investigated the overwintering ability of wheat varieties in Sweden during a period of five years, and obtained a strict correlation between hardiness so estimated and the quantities of soluble carbohydrates. Osmotic pressure determinations by the plasmolytic method gave similar results. Tumanov (42) proved that light is necessary for the hardening-off of wheat seedlings, and that this is because the young wheat plant must photosynthesize in order to build up a sugar reserve. Dexter (8) found the same relationship with regard to carbon dioxide supply. Govorov

(16) distinguishes between spring and winter wheats on the basis of sugars: the former lose glucose more quickly at warm temperatures, the latter gain it more rapidly at low temperatures. Newton and Brown (34) further state that hardy varieties of wheat maintain their sugar reserves during winter better than the less hardy. Mudra (31) asserts that the relative hardiness of winter wheats can be determined refractometrically, or better by sugar determinations.

This body of evidence from workers the world over (and it by no means exhausts the list) seems to prove beyond all doubt the fundamental importance of soluble carbohydrates to cold resistance in cereals. And yet some contradictory results have been recorded. Salmon and Fleming (38) could discover no relation whatever between hardiness of rye, wheat, barley and oats and their sap density during fall and early winter. Tumanov (42) admits that winter vetch (which, however, is not a grain) is an exception among winter annuals, for it is just as hardy as winter wheat and yet possesses only half the amount of soluble carbohydrates. According to Balde (2), Swedish wheat varieties give sugar values which indicate their hardiness, and some German varieties show a similar trend; but it is not possible to obtain significant results by comparing Swedish and German varieties. Gassner and Goeze (14) could discover no hard and fast series of varieties refractometrically. Constantinescu (7) found that varieties of winter barley of different hardiness showed the same decrease in sugar content and in dry matter when hardened off. Also, less hardy varieties showed a greater total sugar content at low temperatures than more hardy ones.

Results with other groups of plants have, on the whole, not proved quite so satisfactory as with winter cereals. Thus, Chandler (6), after an exhaustive series of experiments with plants which kill at relatively high temperatures, concluded that their killing point is lowered slightly when the sap density is increased. This, however, is not true hardening but merely escaping ice formation or, at best, reducing it to a minimum by the possession of a sufficiently high cell-sap concentration.

Of greater importance are the results with more hardy plants. Gail (13) describes a rapid increase in the osmotic pressure of five evergreens from mid-July to December and January. Meyer (29), however, asserts that the increased osmotic pressure of the pitch pine is insufficient to account for the degree of cold resistance developed. Harvey (17) comes to the same conclusion with regard to cabbage. Hildreth (18) found a strikingly similar increase in sugars from fall to winter in the Duchess and Jonathan apple varieties, and though the former is much hardier than the latter, the difference in sugar content during the winter is small. Non-electrolytes in spruce were shown by Lewis and Tuttle (22) to increase from December to March. In *Pyrola*, on the other hand, there was a steady decrease from December to June. Weimer (44) could find no correlation between hardiness and the freezing point of alfalfa. Rein (35) asserts that the cold death-point does not depend at all on osmotic pressure. But he worked with very dissimilar and

unrelated species, many of which were water plants, and these, Lidforss (26) had shown, do not undergo the same changes of starch into sugar as do land plants.

It is, of course, possible that some of the unfavorable results were due to faulty technique, especially in the method used to extract the juice. Yet the negative data are too numerous to be lightly waved aside in this manner. One is therefore forced to conclude that the relation between osmotic pressure and cold resistance is not always demonstrable. Even Lidforss, the original and most vehement protagonist of the role of soluble carbohydrates in cold resistance, points out that some plants (*e.g.*, the sugar cane and the sugar beet) are quickly killed by light frosts in spite of their high sugar concentrations. On the other hand, he admits that bacteria and mosses are examples of those individuals which are cold resistant without any considerable sugar content.

Recently, some investigators have studied the effect on hardiness of artificial increases in osmotic pressure. Åkerman (1) allowed red cabbage cells to deplasmolyze in a molar solution of erythrite, thus increasing their osmotic pressure about 150%. These cells were able to withstand a temperature 3° C. lower than when untreated. Kessler (1935) similarly allowed cells to take up glycerine but found no change in cold resistance. Iljin (19) repeated and confirmed Åkerman's experiments. Dexter (9) allowed cabbage leaves to take up sugar and obtained an appreciable increase in osmotic pressure and cold resistance. However, the hardiness developed was much less than that accompanying a similar natural increase in osmotic pressure. The same was true in Åkerman's results.

Even here, then, there is agreement among the three investigators who used cabbage seedlings, but disagreement by the one using other species (*e.g.*, *Saxifraga*). At any rate, experiments of this type are not sufficient in themselves. It is first necessary to show that osmotic pressure is correlated with hardening under natural conditions.

With the whole subject in an unsettled condition, more information is obviously needed. In the following investigation a considerable number of plants were tested in the hardened and unhardened condition to determine whether they showed a correlation between cold resistance and the osmotic pressure of the cell sap, and further to ascertain whether this factor was of any importance in differentiating species or varieties with respect to hardiness. Especial attention has been paid to seasonal changes in trees, which, on account of their full exposure to low temperature have to possess a greater power of resistance than herbaceous plants.

Methods

Two methods are practicable for the determination of osmotic pressures in plants, namely the cryoscopic and the plasmolytic. Although the latter yields values that are slightly high, both methods, if properly used, give comparable results. The choice should therefore depend primarily on the type of investigation being conducted.

Yet with few exceptions (1, 26) only the cryoscopic method has been used in cold resistance studies. As a result of this, seasonal determinations have been confined to evergreens, from the leaves of which sufficient juice can be obtained at all seasons. This neglect of deciduous woody plants leaves an important gap in the data. Since part of the following research has been conducted to remedy this deficiency, the plasmolytic method was adopted as most convenient.

Besides eliminating the difficulty of obtaining a proper sample of juice, the plasmolytic method is well suited to this type of investigation in other respects. On account of the small size of the sample required, the same plant can be used for all the seasonal determinations. Samples can also be obtained from the same level in a tree without exhausting the supply. This eliminates the variability that exists between individuals and between different altitudes in each. Furthermore, the corresponding tissue can be tested in all cases, so that there is no danger of mixing one tissue in which starch is stored with another in which it is all hydrolyzed.

The validity of the plasmolytic method as a measure of osmotic pressure is questioned by some. The usual objection is that the solute used may penetrate the cell and so raise its concentration, or it may alter the cellular permeability and allow exosmosis of solutes (3). These criticisms are undoubtedly well founded in the case of potassium salts, which have frequently been used as the plasmolytic agent. In the following investigation, however, calcium chloride was always employed. As shown by Fitting (12) and many others, calcium salts do not penetrate. Scarth (39), in fact, found that calcium prevents the penetration of other ions. It can, therefore, be expected to reduce exosmosis to a minimum. Nevertheless, to be certain, sections were tested before and after a six hour period in calcium chloride. No change in osmotic pressure could be detected.

There still remains the question of the accuracy which can be attained with the plasmolytic technique. Osmotic pressure can be determined cryoscopically to .01 atm.; but this degree of accuracy is fictitious as applied to the plant. Thus, Walter (43), admits that certain errors are involved, and he therefore expresses his results only to the first decimal. Fitting (12) attained the same degree of accuracy with the plasmolytic method. This, however, is possible only with very favorable material. With the cells used in the following investigation, determinations could be made only to 0.01 *M* calcium chloride. The error was not more than about 5%.

Procedure

Sections were first allowed to stain for half an hour in a hypotonic solution of 0.15 *M* calcium chloride containing 5 p. p. m. of neutral red. In the few cases where this proved hypertonic a weaker solution was used. Neutral red is a distinct aid in determining incipient plasmolysis. Since only living cells are stained by this dye, it removes the danger of mistaking dead cells

with coagulated protoplasm for plasmolyzed living cells, or empty cell walls for unplasmolyzed living cells. Furthermore, the slightest incipience shows up sharply.

The calcium chloride is a further aid to clear observation, since it prevents the cell wall from becoming stained. A series of calcium chloride solutions were used, varying in strength from 0.05 molar to molar, and all containing 5 p. p. m. of neutral red. The same solutions remained unchanged for several months. This is an obvious advantage over sucrose or other standard organic substances, both as a time-saver and for the sake of uniformity. Minute growths of fungal hyphae do eventually appear, even in the strongest solutions, but since no nutrient is available the growth is exhausted as soon as the food reserves in the spore are used up. This minute growth cannot affect the concentration or utility of the solutions. From time to time, however, they were checked against other solutions and no alteration in the concentration could be detected. Their constancy was further shown by the fact that with the different series of solutions used throughout a period of four years, unhardened, healthy cabbage seedlings always showed the same osmotic pressure, 0.17 to 0.18 *M* calcium chloride.

The osmotic pressure in atmospheres was calculated from the freezing points (International Critical Tables) by use of the formula

$$\text{O.P.} = 12.06\,\Delta - .021\Delta^2 \quad (23).$$

Osmotic Changes Associated with Hardiness

The plant material investigated is, for the sake of convenience, divided into two groups: (a) Herbaceous plants, (b) Woody plants.

(a) *Herbaceous Plants:* Seedlings were grown in the greenhouse until large enough for use (usually at the age of one to three months). In this state they were, of course, unhardened. These were subsequently subjected to a low temperature (5° C.) in a cold chamber cooled with methyl chloride and having continuous artificial lighting. The latter was supplied by four 200 watt lamps, suspended outside the chamber about two feet above the plants.

The degree of cold resistance developed in cabbage seedlings (variety Jersey Wakefield) by a five-day hardening period was readily determined. Together with a control lot of unhardened plants, they were frozen for twelve hours at temperatures ranging between about −2° and −5° C. All the seedlings were then transferred to the greenhouse and left there for two weeks before estimating the injury (*i.e.*, the percentage of dead leaves). As shown in Table I, the unhardened plants always

TABLE I

RELATIVE FROST RESISTANCE OF HARDENED AND UNHARDENED CABBAGE PLANTS

Age, days	Temp. for 12 hr., °C.	% injury	
		Unhardened	Hardened
60	−1 to −4.5	80	10
49	−1 to −4	95	0
75	−1 to −4.5	100	30
57	−1 to −4	70	15
64	−2 to −6	100	25
73	−2 to −4.5	95	10

suffered between 80 and 100% injury, whereas the hardened ones showed an average of 15%. Each result in the table is an average of nine plants. The 20% injury in the case of the hardened plants was always confined to the lowest one or two leaves.

The time factor is of prime importance in the development of cold resistance. Day by day the plant becomes more hardy until the maximum is reached. This gradual increase offers an ideal opportunity for determining the relation between hardiness and osmotic pressure.

For this purpose, some two dozen potted cabbage seedlings were placed in the cold chamber at 5° C. At daily intervals a leaf was removed from each of six plants and the osmotic pressure determined on longitudinal sections of the petiole. The cells examined were those of the chlorenchyma and pith. These two tissues never differed appreciably in cell sap concentration.

Fig. 1 shows that the osmotic pressure increased steadily during the hardening period.

Two clover varieties—a single cut and a double cut—were similarly hardened and tested for osmotic pressure with the same results. Though the former is supposedly more hardy, it did not exhibit any greater increase in osmotic pressure than the latter (Fig. 1).

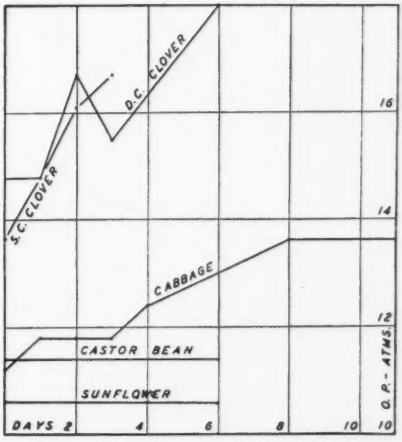

FIG. 1. *Effect of "hardening" temperature (5° C.) on osmotic pressure of hardy (cabbage and clover) and tender (sunflower and castor bean) herbaceous plants.*

Now, cabbage and clover are hardy plants. That is to say, they are able to resist freezing temperatures when in the hardened condition. There are other species, however, which under no conditions will become frost resistant. The question then is: Do the latter show any increase in osmotic pressure as a result of exposure to low temperatures? Sunflower and castor bean seedlings (which belong to this group) were subjected to a temperature of 0° to 5° C. and then tested for osmotic pressure. No change occurred after six days (Fig. 1).

(b) *Woody Plants:* Cabbage and clover, though relatively cold resistant, do not overwinter in the province of Quebec unless well protected. The attempt was therefore made to find out whether the very hardy woody plants exhibit changes in osmotic pressure in relation to hardening or dehardening.

Determinations were made on longitudinal sections of the cortex tissue. Terminal growth was always used. The first tests were of a preliminary nature. Twigs were taken indoors during late winter and placed in a beaker

of water for some days. The comparative osmotic pressures before and after the loss of hardiness undergone in this way are shown in Table II. A marked drop is evident in all cases.

An interesting fact about the twigs dehardened in the laboratory is that little or no starch is observable in the cortex cells. Yet the same cells are full of starch when dehardened naturally. Since the twigs taken into the laboratory were very short (about 3 inches long) much less reserves than normal were available for the increased metabolism of the bursting buds, and this caused a considerable drain on the carbohydrates, thus preventing the accumulation of starch which usually occurs on loss of hardiness.

TABLE II

CHANGES IN OSMOTIC PRESSURE OF TWIGS DURING LOSS OF HARDINESS AT ROOM TEMPERATURE

Species	Date	Days at room temp.	O.P.
Hydrangea paniculata	Feb. 23/34	0	27.3
		4	18.6
		7	14.9
		11	14.9
Caragana arborescens	Mar. 14	0	30.2
		15	14.3
Picea pungens	Mar. 16	0	21.2
		11	18.0
		31	13.7
Pterocarya rhoifolia	Mar. 23	0	36.1
		21	19.9
Catalpa hybrida	Apr. 11	0	28.0
		5	14.9
		12	13.0
Aesculus hippocastanum	Apr. 19	0	21.9
		29	12.4

In order to get a more complete picture of the relation between osmotic pressure and hardiness in woody plants, seasonal determinations were made on several species throughout the year. Since they had survived the severe test winter of 1933–34, none of the plants investigated could be considered tender, and consequently it was impossible to make any comparison between species or varieties at the two extremes of hardiness.

Four apple varieties were chosen. Hyslop (a crab) and Hibernal are the two hardiest, and Delicious and Milwaukee the two tenderest grown at Macdonald College. Since no really tender varieties can overwinter there, even the last two possess a considerable degree of hardiness. Further, the individual specimens of these two varieties which furnished the material were necessarily the hardiest of their group, since all the more tender ones had been killed off by the winter of 1933–34. Thus, the Delicious used was the lone survivor in a row of its kind. Almost all the Milwaukee trees had also succumbed. Yet, though these trees were the hardiest in their respective varieties, they had suffered considerably in 1933–34. All the fruit buds and the terminal growth had been killed. They, therefore, did not flower during the spring of 1934, whereas the Hyslop and Hibernal trees both flowered and fruited abundantly. Presumably, this again would tend to reduce the difference in hardiness between the two groups during 1934–35. Finally, the

tissue investigated—the cortex—is the hardiest of all tissues. In spite of all these conditions which tended to reduce the differences in hardiness between the two groups, there can be little doubt that marked differences still existed. Only current growth was used—the kind that killed on the Delicious and Milwaukee in 1933–34 but was unharmed on Hyslop and Hibernal. Determinations were made at intervals of about three weeks throughout the year from October 1934 to October 1935.

Five species of ornamentals were also tested. These were chosen primarily because of the relatively large size of their cortical cells. This facilitated accurate determinations of osmotic pressures. All the native trees examined possessed cells too small for reliable measurements.

The tendency in all cases is the same (Fig. 2)—a minimum O.P. in May and June followed by a gradual rise to a maximum sometime about October,

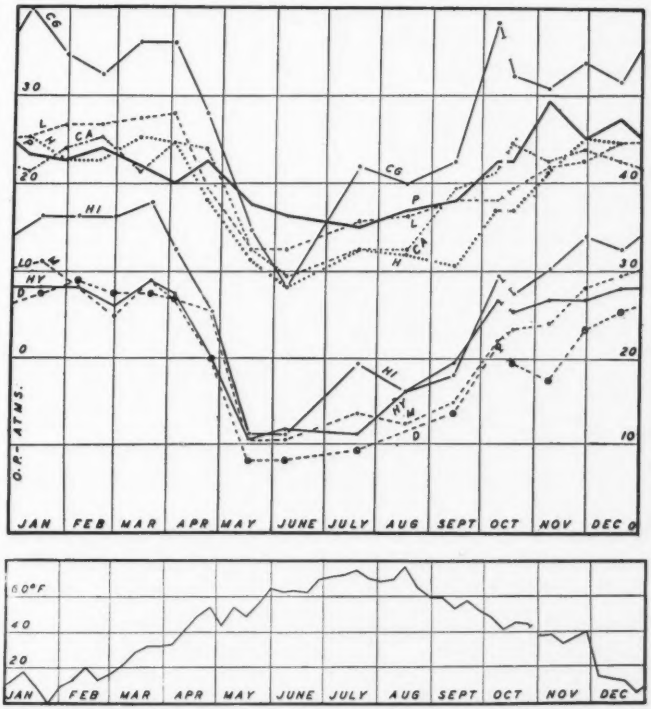

FIG. 2. *Seasonal changes in osmotic pressure of hardy woody plants, determined on cortical cells of current growth, Oct. 1934–Oct. 1935. Lower chart, average weekly temperature.*

Upper Curves

CG = Caragana arborescens
L = Liriodendron tulipifera
CA = Catalpa hybrida
H = Hydrangea paniculata
P = Picea pungens

Lower Curves (apple varieties)

HI = Hibernal } *hardy*
HY = Hyslop }
M = Milwaukee } *less hardy than*
D = Delicious } *above*

this maximum being retained until April. Here a relatively sharp drop occurs to the minimum in May. The difference between the rate of increase and decrease in osmotic pressure is marked. The gradual rise during summer is obviously primarily due to a slow accumulation of assimilated carbohydrates, though it may be partially caused by drought. (This may account for the sudden mid-July rise exhibited by *Caragana* and Hibernal.) The autumn rise, however, (from mid-September onward) must be mainly if not solely due to a hydrolysis of starch into soluble carbohydrates. Thus, tests for starch during winter were always negative. The sharp drop in osmotic pressure during April is due to a reconversion of the soluble carbohydrates into starch, and this is followed by a further drop to its minimum in May, owing to the drain on the stored carbohydrates caused by the rapid spring growth.

The one evergreen, *Picea*, gives an interesting contrast to the rest. Though it also shows a minimum and maximum, the difference between the two is far less. The maximum is similar to that of the deciduous species but the minimum is about double theirs. Owing to the conifer's ability to photosynthesize just as soon as the temperature rises sufficiently, and also to its less vigorous spring development, the drain on its stored carbohydrates is much less.

The difference between the maxima and minima is astonishingly great. In *Caragana* the minimum is about $7\frac{1}{2}$ atm., the maximum about 40, an increase of over 400%. The extremely high winter osmotic pressure of *Caragana* is interesting in view of its great cold resistance. Judging from its native habitat, it is by far the hardiest of the lot. Not only is its maximum osmotic pressure easily the highest, but it is also reached earliest. In the apples, too, the hardier varieties apparently reach their maximum osmotic pressure sooner than the less hardy. This may indicate an earlier "ripening off" in the case of the more hardy plants, a habit which would be very advantageous in sudden, early "test winters".

During the depth of winter, however, no strict correlation between hardiness and osmotic pressure is evident in the apple varieties. Hibernal, it is true, possesses the highest of all the four. Hyslop, on the other hand, has almost exactly the same osmotic pressure as the two more tender varieties.

Discussion

In the species examined, osmotic pressure is undoubtedly correlated with hardiness. Thus, the eight hardy species tested underwent an increase in osmotic pressure during assumption of cold resistance, whereas the two tender species did not change when exposed to "hardening" temperatures. Furthermore, the two semi-hardy (herbaceous) species had maximum osmotic pressures of only one-third to one-half as great as those found in the six hardy (woody) species. Also among the latter, the hardiest single species possessed the highest concentration and was first to reach its maximum. Among four apple varieties tested the highest osmotic pressure was found in one of the

two hardiest, while the other hardy variety did not differ from the two more tender ones. However, both hardy varieties reached their maxima before the tender ones.

These facts support the view that osmotic pressure is one of the factors involved in cold resistance.

As to its operation, the effect of increased osmotic pressure with regard to freezing is not merely to depress the actual freezing point but also to reduce the amount of ice which forms at temperatures below the freezing point, the latter effect being much the more important as a protection to the cell. It was observed during plasmolysis tests of tree tissues that the reduction in volume of the cells (which is the amount of water withdrawn from them) in solutions of varied strength falls far short of theoretical expectation. The same must apply to the amount of ice which is formed at freezing. In other words, the osmotic pressure of the cells at incipient plasmolysis is not, in such cases, a true measure of their power of resisting ice formation at any and all temperatures. It is necessary to know the osmotic value of the cells at various degrees of plasmolysis in order to correlate temperature with ice development. The next step, therefore, is a study of this relation and of the factors concerned.

The Non-osmotic Fraction of the Cell

If the cell represents nothing more than an osmotic membrane surrounding a solution, then it will obey Boyle's law in as far as that applies to solutions, so that PV will be approximately constant. Thus, if a cell is plasmolyzed by a twice isotonic solution, it should be reduced to half its normal volume. In the case of cabbage this relation was found to hold. Cylindrical cells were chosen and the relative volume of the protoplasts estimated, using the formula: $g = \frac{l - \frac{1}{3}d}{h}$. In both the hardened and unhardened condition the volume of the cell was halved in a twice isotonic solution (Table III). This establishes the fact that the cell sap contains no appreciable amount of solid material that is not osmotically active, and that the force by which it holds water is purely osmotic.

TABLE III

CHANGE IN VOLUME OF CABBAGE CELLS WITH CHANGE IN OSMOTIC PRESSURE
AVERAGE OF 10 CELLS

Seedling	Unhardened		Hardened 4 days		Hardened 7 days	
	Isotonic $CaCl_2$ (mols)	*g in $2i$ $CaCl_2$	Isotonic $CaCl_2$ (mols)	g in $2i$ $CaCl_2$	Isotonic $CaCl_2$ (mols)	g in $2i$ $CaCl_2$
1	0.17	0.50	0.19	0.48	0.20	0.51
2	0.18	0.50	0.21	0.50	0.20	0.48
3	0.16	0.48	0.19	0.51	0.20	0.46
Average	0.17	0.49	0.20	0.50	0.20	0.48

*g = *Höfler's "degree of plasmolysis", i.e., that fraction of its normal volume occupied by the plasmolyzed cell.*

Such an ideal relation is by no means always present. Thus, previous results with onion cells (24) showed that the simple formula $PV = K$ does not hold. It is therefore necessary to use the modified Van't Hoff formula $P(V - x) = K$, where x represents that fraction of the cell volume which does not vary directly with P. From this, $x = \frac{V_1P_1 - V_0P_0}{P_1 - P_0}$.

It is a matter of controversy whether the existence of x in cells is due almost entirely to the presence of non-osmotically active solids or whether colloidally bound (non-solvent) water is also an important factor (27). The question may be decided directly where it is possible to measure accurately the dry weight of colloidal material in the cell. In the case of plant tissues an indirect approach must be used. If x is merely solid matter, its value will be constant for all values of P. On the other hand, if bound water enters into it, then x will vary with P, since bound and free water are in equilibrium. Determinations of x should then be useful in discovering the existence of "bound water" and in comparing its amount in hardy and non-hardy plants.

We have already seen that the value of x is zero, and that therefore there is no bound water in the cell sap of cabbage, whether in the hardened or unhardened state. It is, of course, impossible to generalize from this one case. Furthermore, this tells nothing about the protoplasm itself, since it occupies an insignificant fraction of the large cabbage cell.

An investigation was therefore made on the cortex cells of *Catalpa* twigs in the fully hardened winter condition, and also after they had lost their hardiness by being kept at room temperature. Table IV shows that there is a considerable difference in the value of x, which is 50% greater in the hardened than in the unhardened cells.

TABLE IV

COMPARISON OF x IN CORTICAL CELLS OF HARDENED AND DEHARDENED *Catalpa* TWIGS

	O.P. (atm.)	g in $2i$	x (% of normal volume)
Hardened	18.6	.71	41
Dehardened	11.2	.64	28

Since the osmotic pressures of the hardy and non-hardy cells are not the same, the values of x, if due to bound water, are not strictly comparable, for bound water will vary with the osmotic pressure. The question now is: What is the difference between the values of x when determined under the same osmotic pressures?

To answer this, measurements of cell volume were made in plasmolyzing solutions of different concentrations. Spherical cells were chosen, since cylindrical ones lose their regularity in the very high concentrations here employed. Table V shows the values obtained. The volume of the cell is given both in percentage of its measured volume in 1.0 M dextrose and of its normal volume as found by extrapolation of the volume curve. The value of x is also calculated on these two bases.

TABLE V

COMPARISON OF x IN HARDENED AND DEHARDENED *Catalpa*. AVERAGE OF 16 CELLS

Conc. dex. (mols)	Relative cell vol.				x			
	Hardened		Non-hardened		Hardened		Non-hardened	
	% volume in *M* dex.	% normal volume	% volume in *M* dex.	% normal volume	% volume in *M* dex.	% normal volume	% volume in *M* dex.	% normal volume
1.0	100	85	100	77				
					58	49	50	39
1.5	83	70	80	62				
					47	40	44	34
2.0	72	61	69	53				
					41	35	41	32
2.5	63	53	61	47				
					55	47	38	29
3.0	61	52	55	42				

We see that x varies with P and is therefore at least partially due to "bound water". The cold-resistant cells again are characterized by a higher value of x.

Catalpa cells have unusually thick protoplasm layers (Fig. 3) and since the cells are small (compared with cabbage), the percentage of the volume occupied by it is large, about 50%. The possibility suggested itself that the

FIG. 3. *Plasmolyzed Catalpa cortical cells showing thickness of protoplasm layer. Vacuole stained with neutral red.*

greater part of x might be localized in the protoplasm. Measurements were therefore made of the thickness of the protoplasm layer in the various concentrations of the plasmolytic agent, as well as of the diameter of the cell as a whole. The relative volumes of x calculated from these measurements are given in Tables VI and VII.

TABLE VI

COMPARISON OF x IN HARDENED AND DEHARDENED *Catalpa* VACUOLES. AVERAGE OF 16 CELLS

Conc. dex. (mols)	Relative vac. vol.				x			
	Hardened		Non-hardened		Hardened		Non-hardened	
	% vac. vol. in *M* dex.	% normal cell vol.	% vac. vol. in *M* dex.	% normal cell vol.	% vac. vol. in *M* dex.	% normal cell vol.	% vac. vol. in *M* dex.	% normal cell vol.
1.0	100	32.8	100	32.8				
1.5	90	29.5	80	26.1	75	24.5	50	16.6
2.0	82	26.7	69	22.8	63	20.5	44	14.5
2.5	73	23.8	63	20.8	51	16.6	48	15.8
3.0	71	23.0	58	19.1	65	21.2	44	14.5

TABLE VII

x IN HARDENED AND DEHARDENED *Catalpa* PROTOPLASM. AVERAGE OF 16 CELLS

Conc. dex. (mols)	Relative prot. vol.				x			
	Hardened		Non-hardened		Hardened		Non-hardened	
	% prot. vol. in *M* dex.	% normal cell vol.	% prot. vol. in *M* dex.	% normal cell vol.	% prot. vol. in *M* dex.	% normal cell vol.	% prot. vol. in *M* dex.	% normal cell vol.
1.0	100	51.8	100	43.9				
1.5	79	41.0	79	34.5	48	25.0	48	21.0
2.0	67	34.5	68	29.8	39	20.5	43	18.7
2.5	58	30.0	58	25.7	36	18.5	38	17.0
3.0	56	28.8	50	22.2	50	25.5	27	12.3

These results show that the difference in x lies not so much in the protoplasm, which might be expected, as in the vacuole.

Seasonal investigations of x were made in *Catalpa* and *Liriodendron*. In both cases, about a 50% drop in x accompanied the natural loss of hardiness which occurs during spring (Table VIII).

TABLE VIII

SEASONAL CHANGES IN x. AVERAGE OF 10 CELLS

Date	*Catalpa*			*Liriodendron*		
	O.P.	*g* in 2*i* $CaCl_2$	x	O.P.	*g* in 2*i* $CaCl_2$	x
Mar. 12	22.6	.72	42	25.2	.71	37
Mar. 31	21.9	.70	40	21.9	.68	36
Apr. 20	23.2	.75	52	19.3	.62	24
May 11	14.3	.60	20	17.4	.60	21
June 1	12.4	.65	29	13.0	.53	6
June 22	9.4	.57	13	14.3	.58	16

There is one main difference between cells dehardened in the laboratory and those naturally dehardened. In the former, as previously mentioned, little or no starch appears; in the latter, considerable amounts occur. This would complicate the seasonal change in the value of x. Fortunately, however, *Catalpa* is an exception, for no starch was found in its cortical cells even during June.

It is interesting to compare the theoretical cell volume under a certain degree of frost of hardened and unhardened cells, respectively, as calculated from the osmotic pressure and value of x. The temperature at which unhardened twigs are quickly killed will serve as point of comparison.

Unhardened *Catalpa* twigs (collected in June) were exposed to a temperature of $-6°$ C. for six hours. They were removed from the cold chamber and set in water. Next morning the leaves and young shoot were seen to be dead and blackened. The one-year twig showed injury in two ways:—First, the water in the beaker was colored light yellow, owing to exosmosis from dead cells; second, where the cortex was cut, it was seen to be of an unnatural dark-green color. Sections of the cortex showed that most of the cells were dead, at most only 10% staining with neutral red and exhibiting plasmolysis in a hypertonic solution.

Thus, a temperature of $-6°$ C. was capable of killing most of the cortex cells. Now, since the osmotic pressure of these cells was found to be 9.4 atm. and the value of x, 15%, the following calculations are possible.

$$\text{At } -6° \text{ C., conc. of sap} = \frac{6}{1.86} = 3.22\ M \text{ dextrose}$$

$$\text{Normal conc. of sap} = 9.4 \text{ atm.} = 0.39\ M \text{ dextrose}$$

$$\therefore \text{At } -6° \text{ C., vol. of sap} = \frac{39}{3.22} = \tfrac{1}{8} \text{ of normal}$$

But since $x = 15\%$, only 85% of the cell volume is affected (assuming that x is non-soluble solids and therefore not reduced by the increased pressure).

$\therefore$ Amt. of water removed is at least $= \frac{7}{8} \times 85 = 75\%$ of cell,
i.e., cell is reduced to 25% of its normal volume.

Now the same *Catalpa* cortical cells have a maximum winter value of $x = 40\%$ and an O.P. of 16 atm. Therefore if x were irreducible by ice formation, minimum volume of cell at any temperature would be $\frac{2}{5}$ of its normal volume. But it has been shown that x falls as osmotic pressure rises, the value of 40% in near ($1\frac{1}{2}$) isotonic concentrations being reduced to 25% in 3 M dextrose (approximately the concentration reached at $-6°$ C.).

$$\text{At } -6° \text{ C., then, conc. of sap} = 3.22\ M \text{ dextrose}$$

$$\text{Normal conc. of sap} = 16 \text{ atm.} = 0.66\ M \text{ dextrose}$$

$$\therefore \text{At } -6° \text{ C., vol. of sap} = \frac{.66}{3.22} = \tfrac{1}{5} \text{ of normal.}$$

But since $x = 25\%$ at $-6°$ C., only $\frac{3}{4}$ of the cell volume is affected.

$\therefore$ Amt. of water removed $= \frac{4}{5} \times \frac{3}{4} = 60\%$ of cell,
i.e., cell is reduced to 40% of its normal volume.

At 25% the winter value of x seems to be approaching a minimum. Assuming that further dehydration is not produced at any temperature, then it would be impossible for the cell to contract to the point at which it was killed in the unhardened state—for even below the eutectic point the soluble solids occupy some space to be added to that of insoluble solids and bound water.

Discussion

Osmotic pressure, apart from bound water, was discussed on page 273. An estimation of the non-osmotically active portion of the cell (designated as x) constitutes a valuable method of determining the role of "bound water" in cold resistance. In fact, it is the only technique so far employed on living tissue. Newton's pressure method is in some degree an exception, but it is not a true measure of "bound water" (see 43); other methods cause injury.

Cabbage cells both in the hardened and unhardened condition possess no measurable portion that is non-osmotically active. There is, therefore, no "bound water" in the cell. This is in opposition to the results of investigators (4, 10, 36) who used other methods on the dead products of this plant.

In *Catalpa* and *Liriodendron* cells, on the other hand, the non-osmotically active fraction is large, being greater in the hardened than in the unhardened condition. Furthermore, since the value increases with increase in volume, *i.e.*, water-content, of the cell and since "bound water" increases with free water (within limits), the inference is that x is partially due to "bound water".

A separate estimation of x in the protoplasm and vacuole of *Catalpa* reveals that x, while its actual amount is about the same in both, occupies a relatively larger fraction of the vacuole, and that it is here that the main difference in x between hardened and dehardened cells—at least when dehardened in the laboratory—resides.

During the early part of May, x in *Catalpa* drops to about one-half of its winter value. This drop occurs before any appreciable growth, and therefore it cannot be due to the non-osmotically active solids being used up in growth, as is probably the case later. Another possibility is that these solids may be hydrolyzed to soluble substances, but unless it is counteracted by a concomitant decrease in "bound water" this is unlikely, in view of the marked drop in osmotic pressure coincident with the decrease in x.

The most plausible explanation, then, is that the seasonal drop in the value of x at this stage is due to a decrease in "bound water".

The high mid-winter pressures of woody plants are therefore not merely due to an increase in osmotically active units by hydrolysis of starch to sugar, but are partly caused by an increase in "bound water".

These results give positive evidence of the value of the much disputed "bound water" in cold resistance. However, the usual explanation of its role as preventing the dehydration of protoplasm does not appear to hold, for the increase in non-solvent space occurs less in the protoplasm than in the vacuole. When ice forms inside the cell, its site is the vacuole, not the

protoplasm (5). The cell sap is the vulnerable part of the cell and the function of the increase in "bound water" as well as of osmotic substance in the vacuole may be to prevent the freezing of the sap.

But even if ice remains external to the cell, the non-solvent space may be supremely important. Being non-solvent the "bound water" is, of course, valueless in preventing concentration of the sap, which some regard as the immediate cause of frost injury, but it may serve a purpose in reducing the amount of ice formation or in modifying the degree of cell shrinkage, both of which are possible sources of mechanical injury. Our calculations show that at a temperature of $-6°$ C., which is lethal to unhardened *Catalpa* cells, the volume of ice in the tissues is 75% of the original cell volume, while the volume of the cells themselves shrinks to 25%. At the same temperature with hardened tissue the volume of ice is 60% and the cells 40%. Still more significant is the calculation that in the latter, no matter how low the temperature, the ice volume can never rise nor the cell volume fall to those which are reached with fatal effect at only $-6°$ C. in unhardened tissues. This illustrates rather forcibly how important a role hydrophilic colloid in the sap—in conjunction with osmotic pressure—plays in the frost resistance of the cells which have to endure extremely low temperatures; but moderate resistance, as we have seen, is attained without this factor, while its presence in considerable degree in unhardened *Catalpa* cells does not make them as frost resistant as those of hardened cabbage, in which it is immeasurably small. Evidently, other factors are involved and these will be dealt with in subsequent papers.

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STUDIES ON THE BIONOMICS AND CONTROL OF THE BURSATE NEMATODES OF HORSES AND SHEEP

II. TECHNIQUE[1]

BY I. W. PARNELL[2]

Abstract

This paper describes the standardization of the technique that has been evolved to facilitate the comparative testing of various substances that can be added to manure with the object of effectively destroying the free-living stages of bursate nematodes. Methods of controlling the experiments are described and the significance of the results discussed.

The unexpected lethal effects of urine on the eggs and larvae of bursate nematodes reported in Part I. of this series of papers (13) gave hope that other substances with a similar effect might be found, which could be added to feces and which would be of value in increasing the fertilizing properties of the manure or would be easily and cheaply obtained by the farmer. It also opened up the possibility of similarly treating human excrement in countries where hookworm disease is endemic and where lethal effect is more important than manurial accretion; a very large number of possible chemicals could be tested for this purpose. Moreover, further tests on urine were necessary. This made it essential that a standardized technique should be evolved, not only for the actual tests but for the large number of controls necessary. The present paper describes the technique that was adopted and the methods that were used to control the experiments. Future papers will describe further experiments with urine, with nitrogenous and with non-nitrogenous artificial fertilizers, with various chemicals used to control plant eelworms and insects, with disinfectants and with some other miscellaneous chemicals which appeared to have potential applications.

To enable comparisons to be made between the different substances tested, the technique already described in Part I (13) of making the cultures in cheese cloth which could be suspended in funnels for the extraction of the larvae, has been used throughout. This method of culturing the feces in a cheese-cloth bag allows excellent drainage, yet the lightly covered sealer retains sufficient moisture for many weeks and contains sufficient oxygen for development of the larvae. Maplestone (11) has pointed out that it is not necessary to trap hookworm larvae if they are allowed to develop under suitable con-

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ditions, and that Lane's (7) traps are only necessary when there is bad drainage, such as in a Petri dish, which causes unsuitable conditions from which the larvae migrate. Sclerostome larvae in the sealers have seldom been seen as high as the neck, and few, if any, reach the lid, although it, as is the sealer, is always washed into the funnel.

All cultures have been made from horse feces collected in a single stable of adult heavy-draft horses, all on a similar diet; when feces from more than one horse have been used or when many cultures were made at the same time, more than one "control" has been made.

All cultures have been kept in a dark constant temperature room at 25° ± 2° C., until the larvae were extracted, except those treated with chemicals that give off gases that are undesirable in a small unventilated room,—chloropicrin, carbon disulphide and calcium cyanide.

The cultures were always made of 40 grams of horse feces weighed when fresh, but no allowance was made for any variation in the consistency of the feces. The variation, owing to the uniform treatment of the horses, was slight. The cultures were always kept for more than nine days to ensure that all larvae had time to reach the infective stage, and be acted upon in that stage. Usually they were kept considerably longer. Lucker (8, 9) Ortlepp (12), Taylor (16), and others have shown that the infective stages are reached in less than a week at temperatures similar to or lower than that at which these cultures were kept.

Two methods have been used to test the action of the fertilizers on eggs or larvae or both in the presence of feces. The method used in treating the cultures to be discussed in the papers that will follow, has been to allow the chemicals to remain in the cultures so that they could act on all stages subsequent to the incorporation of the chemicals. The other method to test the action of the chemical on eggs and free-feeding larvae only, and not on subsequent stages, has been to treat the feces either while fresh (and the eggs therefore undeveloped) or after there has been time for the appropriate amount of development. After the chemical has been in the feces for a sufficient length of time, *e.g.*, 24 or 48 hours (during which time the majority of the larvae would develop to the appropriate stage) the greater part of the chemicals has been removed by washing the cultures in several hundred cubic centimetres of warm water, squeezing them until there was only sufficient water for optimum development, filtering the washing water, and culturing the filter paper in the same sealer as the feces.

It is hoped that a comparison of the various results obtained when incorporating the same quantities of chemicals will demonstrate the most practical stage at which to treat feces with each of the more lethal substances.

When the egg stage, with or without the succeeding stages, has been included in the "treated" period, the feces have been mixed with the chemical, fertilizer, or other substance to be tested, within four hours of being passed.

Solid fertilizers or chemicals have been tested by mixing dry with the feces, and also as solutions if they are soluble in water. Sometimes "solutions"

have been used in strengths above the point of solubility, but for the purposes of these papers these suspensions and solutions are all referred to as solutions. Fluids have been tested undiluted, and also diluted if miscible with water. It will be seen from the tables that in testing any fertilizer or other substance, the cultures were made up with the chemical and feces in a wide range of proportions. Even when these had indicated the lethal percentage necessary, confirmatory tests were made not only with the indicated proportions but also in greater and smaller amounts; the important range was tested in triplicate, the quantities above and below in duplicate. When desirable, cultures were also made well above or below the lethal point, not only for confirmation but for comparison with other fertilizers or chemicals. Unfortunately, the supply of urines, especially stale urines, prevented them being tested quite as thoroughly.

Twenty-five cubic centimetres is more than sufficient to moisten thoroughly 40 grams of fresh horse feces, and with this quantity of fluid, and sometimes even with 20 cc., there is some drainage from the bag of feces to the bottom of the sealer, so that part of the larger quantities of fluid used is only temporarily in contact with the Sclerostome eggs.

Excessive moisture may assist in the prevention of the development of eggs and free-feeding larvae in the centre of a mass of feces, by excluding the oxygen necessary for development. Moisture also alters the bacterial and mycological flora of the feces; the former may be an important food of free-feeding larvae, the latter may be parasitic on them. This flora may also be altered by some of the chemicals tested.

Fungi have been reported which are parasitic on nematodes and which use a three-dimensional net-like structure to capture their prey. Others, described by Drechsler (1, 2) and Sherbakoff (14), capture their prey in constricting loops, or by means of adhesive material on globose protuberances, after which the mycelium grows into the nematodes. It is probable that these fungi, under suitable conditions, may materially reduce a nematode population.

Occasionally the feces were contaminated by free-living nematodes before collection, and with some fertilizers these persisted in cultures in which the Sclerostomes were killed, and so the results are not applicable to free-living species. In a few cases the free-living species may have been predators, but contamination has not occurred sufficiently frequently to influence the conclusions.

To determine the number of larvae which reached the infective stage, the cultures were put into glass funnels, closed at the bottom by a clip on rubber hose. The inside of the sealer was washed into the funnel to collect all larvae which had migrated from the culture, and the funnel filled with warm water. In the funnels the larvae were stimulated to leave the cultures by scooping off or pouring off the top fluid and adding an equivalent amount of hot water nearly every day. Large funnels were used during the extraction in an endeavor to prevent the chemicals acting on the larvae during their

collection. Pouring off the top fluid several times during the process has three major advantages:—

(1) It dilutes very considerably any chemical that has been used to treat the feces.

(2) The added hot water stimulates the larvae.

(3) Emptying and filling the funnel dislodges any larvae on the sides of the funnel more effectively than tapping the funnel, as described by Lane (7).

It has the disadvantage that it washes down any sediment, which would be serious in soil cultures, and thus has added to the difficulty of counting, especially in cultures treated with lime and phosphate and some other chemicals such as cyanamide.

While some dead Sclerostome larvae may float—Hirst (4) has found that hookworm larvae do—the great majority sink, unless supported by the side of the container or by floating foreign matter. Therefore this technique does not lose dead Sclerostome larvae which are outside the culture.

To estimate the number of larvae extracted from a culture the bottom fluid was drawn into a 50 cc. Erlenmeyer flask in which the larvae were left to settle; the top fluid was then discarded and the larvae at the bottom were poured into a square Petri dish which was divided into squares. In dealing with negative cultures or with cultures containing a few to two or three hundred larvae, the whole dish was examined. When there were only a few thousand larvae their numbers were estimated by counting those on a known area of the dish, always at least one sixth of the area. When there were several thousand larvae their number was estimated by shaking them up in 40 cc. or 80 cc. of water, and counting the number in 10 cc. or 20 cc.; in this case, one larva in at least every 24 was counted. When the larvae were exceptionally numerous, *i.e.*, 50,000 or more, the fluid was usually diluted in the same proportions again, in which case the number counted multiplied by 96 gave the number of larvae in the cultures. No culture was considered negative until no larvae could be collected after it had been in the funnel for more than four days at least. Positive cultures were kept in the funnels until the larvae that could be extracted were only a negligible proportion of those already counted; in a few cases this was two or three weeks, since some substances appeared to immobilize rather than kill the larvae. In a few cases, after having been in the funnels for some days, and the majority of the larvae having apparently been extracted, the cultures were broken up and rebagged to ensure that the majority of larvae had left the centre of the culture. More larvae were obtained from the cultures under these circumstances, but never sufficient to alter the significance of the number of those originally collected.

The counting of Sclerostome larvae presents an important difference from the counting of eggs, because the larvae when on a microscopic stage, under the influence of a fairly bright light, become very active; this not only makes them more difficult to count when numerous, but may involve their being

counted twice or missed, when they swim from one row to another. Furthermore fungi or debris occasionally make distribution of the larvae throughout the dish most unequal, since the larvae may very rapidly become caught and form large struggling masses. Therefore, in spite of the fact that the larvae in the cultures have been estimated by carefully counting a large proportion of them and all the results have been checked, the numbers given must be considered as only approximate when the larvae were numerous. However this does not obscure the object of the work—to show the proportion of the various fertilizers or chemicals necessary to kill the free-living stages of Sclerostomes in feces and to suggest their relative strengths as sterilizing agents against the bursate nematode larvae.

To prevent a false impression of the accuracy obtained, the tables in these papers show the number of larvae isolated on the following basis:

1–	10—Number counted.	
10–	20—Even numbers	
20–	40—Nearest number divisible by	5
40–	60—Nearest number divisible by	10
65–	200—Nearest number divisible by	25
200–	750—Nearest number divisible by	50
800–	4,000—Nearest number divisible by	100
4,000–	8,000—Nearest number divisible by	200
8,000–	30,000—Nearest number divisible by	500
Over	30,000—Nearest number divisible by	1,000

In a few cases, especially with active larvae or where there was much debris, even these figures may suggest an unjustifiable accuracy.

Little significance need be placed on the fact that in some cultures a few larvae are found, since when the cultures are tied up in cheese cloth and put into sealers, it is difficult to prevent minute pieces of feces and the eggs therein from adhering to the outside of the culture away from the influence of the fertilizer, or some feces and eggs from being squeezed on to the neck of the sealer when the culture is being put into it. Contamination of the counting dish or of the funnel is also a possible cause of an occasional culture not being completely negative when it should be, since the only glassware it has been possible to sterilize has been the sealers; the funnels, flasks and counting dish have only been washed. Another cause may be that the feces contain only a few eggs of a species resistant to the effects of the chemical being tested. No attempt has been made to determine the percentages of the various species of larvae, but the writer's impression is that the percentage of the *Strongylus* spp. has been lower than that in cultures made by Mahlau (10) in Berlin. It has been noted frequently that the larvae of *Strongylus* spp. are more resistant than those of *Trichonema* spp. to unfavorable conditions, whether chemical or physical.

In the tables showing the effects of the various substances on the numbers of larvae, a letter is used to indicate the condition of the larvae as follows:—

a—Most or all larvae dead.

b—A considerable proportion of the larvae dead.

c—A few of the larvae dead.

d—Much larval debris uncounted.

Although the larvae may have looked perfectly healthy it is impossible to say whether or not they would have been capable of infecting horses. It has been found that when only a few larvae survived in a culture, their life in water in the laboratory tended to be shorter than those from cultures in which many larvae survived, apart from the fact that if an excessive number of larvae are kept in a small quantity of water, they die fairly rapidly.

Each number is followed by a Roman numeral, which corresponds to the number of the control or controls listed in the table of controls for each paper; the same numeral is used for the same control in all the papers, so that not only can the significance of the number be appreciated, but cultures made from the same series can be traced.

The time between the making of the cultures and the extraction of the larvae is shown in weeks or fractions of weeks. The date on which the cultures were made is also shown, since Hornung (6) and Wagner (17) found that the egg count of horses in Germany varies with the season, although the observations of Gogolka (5) are to the contrary.

Tests have been made to determine the probable natural accuracy or inaccuracy of parts of the technique used to prevent any undue significance being attached to the variations in the numbers of larvae obtained.

TABLE I

NUMBERS OF LARVAE OBTAINED FROM CULTURES OF UNTREATED HORSE FECES SHOWING THE NUMBER AND PERCENTAGE OF LARVAE OBTAINED, COMPARED WITH THE ESTIMATED EGG CONTENT

Horse	Day	Number of larvae	Percentage of estimated number of eggs reaching the infective stage
Bay	1st	13,500	57
Bay	2nd	16,000	44
Bay	29th	6,600	14
Bay	30th	26,500	56
Roan	1st	13,500	45
Roan	2nd	12,500	40
Roan	29th	6,600	26
Roan	30th	9,000	43
Grey	1st	6,200	39
Grey	2nd	7,200	37
Grey	29th	2,200	55
Grey	30th	6,000	60

To determine what significance could be put on the numbers of larvae obtained from untreated cultures, egg counts were made of feces from three horses on two consecutive days, the Stoll egg-counting technique (15) being used. At the same time 20-gm. cultures were made up and after 15 days these were baermanned. Four weeks later similar egg-counts and cultures were made with feces from the same horse; the results of both are shown in Table I.

Feces were collected from another horse on two consecutive days, to determine whether there was any significant variation in the numbers of larvae obtained when the feces were cultured broken up or in lumps, and whether there was any significant variation in the numbers of larvae obtained from the feces first passed and those last passed from the horse. On each day, five 40-gm. cultures were made. One culture was made of one large lump, another was partly and a third completely broken up, using the feces that were passed first. From the feces passed last one culture was made of one large lump and another of completely broken up material. The larvae were isolated after culturing for 31 days. Table II shows the numbers of larvae obtained.

TABLE II

NUMBERS OF LARVAE OBTAINED WHEN THE FECES ARE BROKEN UP OR NOT BROKEN UP BEFORE CULTURING, AND THE NUMBERS OF LARVAE OBTAINED FROM THE FECES FIRST PASSED COMPARED WITH THOSE LAST PASSED

	In lumps		Partly broken up	Broken up	
	First passed	Last passed	First passed	First passed	Last passed
1st day	14,500	71,000	43,000	73,000	39,000
2nd day	37,000	44,000	57,000	41,000	45,000

Average per gram: In lumps, 1,040; Broken up, 1,240; First passed, 1,106; Last passed, 1,244.

To determine the efficiency of the larval extraction method nine cultures were twice sterilized by heat, (steam and dry), after which 75 to 100 larvae were poured into three cultures, 700 to 1,000 into three others, and about 10,000 larvae into three more. The larvae were then extracted from these cultures in the ordinary way. From the three cultures which received the smallest number of larvae 12½%, 25% and 45% were recovered; from the three cultures in the second group the recovery was 35%, 35% and 50%; and from the one containing about 10,000 the recovery was 45%, 65% and 90%. All the larvae used were from control cultures previously baermanned, the three 10,000 lots having been extracted a few days before being put back into feces, while the others had been collected a day or two earlier. There is a considerable tendency for larvae to become less active after they have been in water for a few days, unless affected by some stimulus such as a bright light; therefore a smaller percentage than usual would be recovered. Hirst (3), working with hookworm larvae added to various soils, found that approximately 12% were recovered in 24 hours from laterite soil, 17% from fine sand and 82% from gravelly loam with much humus.

To test the effects of the addition of water on the number of larvae obtained from 40 gm. of horse feces, various quantities were added to otherwise untreated fresh feces. Table III gives the number of larvae obtained. This table suggests that the addition of the larger quantities of water decreases the number of larvae which survive.

To supplement Table III details of the results obtained with rabbit urine, "byre fluid" etc. will be given in full in Paper III instead of a brief report of the negative results obtained with them.

TABLE III

QUANTITY OF WATER ADDED TO 40 GM. OF FRESH HORSE FECES, AND NUMBERS OF INFECTIVE SCLEROSTOME LARVAE ISOLATED AFTER CULTURING

	1.0 cc.	2.0 cc.	3.0 cc.	4.0 cc.	5.0 cc.	7.5 cc.	10.0 cc.	15.0 cc.	20.0 cc.	25.0 cc.
	22,000	30,000	22,500	23,000	8,500	20,000	17,000	15,000	3,600	5,200
	5,000	49,000	43,000	47,000	38,000	24,500	37,000	33,000	15,500	900
	38,000	39,000	24,000	4,700	49,000	16,000	18,000	19,000	16,000	7,000
		10,500	40,000	35,000	31,000	5,400	45,000	25,000	20,500	20,000
		24,500	31,000	23,500	3,500	1,800	13,000	1,100	10,000	3,500
		900	26,500	6,600	22,000	27,500	31,000	15,500	5,000	22,500
		12,500	23,500	22,000	9,000	42,000	6,800	17,000	22,500	33,000
		43,000	39,000	36,000	13,500	22,500	4,000	25,500	32,000	13,500
		42,000			30,000		9,000	52,000	15,500	8,000
		53,000			42,000		40,000	5,800	35,000	26,500
					17,000		34,000	15,000	27,000	2,400
					62,000		30,000		12,000	19,000
							13,000			27,500
										13,000
										39,000
										10,000
										3,700
										3,500
										7,800
Average no. of larvae isolated from cultures to which water was added	21,500	30,000	31,000	24,500	27,000	20,000	23,000	20,500	19,000	14,000
Average no. of larvae isolated from the untreated control cultures	39,000	27,000	29,500	29,000	30,000	32,000	25,500	24,500	37,000	28,000

In addition to the results tabulated, one culture treated with 0.5 cc. of water yielded 12,500 larvae, compared with 13,000 in the control; two treated with 12.5 cc. of water yielded an average of 36,500 compared with 47,000 in the controls. Twelve treated with 10 cc. water, including six in which chaffed sterilized straw was incorporated, but for which no dry controls were made, yielded an average of 21,000 larvae, while six similar cultures treated with 15 cc. of water yielded an average of 11,500 larvae.

TABLE IV

RATE OF COLLECTION OF LARVAE DURING THE ISOLATION PROCESS

Time, hr.	9 in. funnel, %	8 in. funnel, %	6 in. funnel, %	Remarks	Average of the 3 funnels, %	Average rate per hour, %
1	50.0	48.2	42.55	Natural and artificial light	46.75	46.75
2	11.11	13.4	8.5		11.15	11.15
3	2.22	5.54	2.55		3.67	3.67
4	2.22	2.77	1.49	Hot water added to funnels immediately after 4th hour.	2.06	2.06
5	6.53	4.06	9.7		6.53	6.53
6	8.88	8.93	4.84		7.53	7.53
6–8	5.83	4.3	1.54		3.76	1.88
8–10	0.83	1.47	0.96		1.13	0.56
10–12	0.35	0.4	0.69	Dark	0.49	0.25
12–14	0.11	0.4	0.48	Artificial light	0.35	0.17
14–16	0.28	0.4	0.42	Dark	0.38	0.19
16–20	0.17	0.36	0.64	Artificial light	0.4	0.1
20–24	0.35	1.3	1.27	Natural and artificial light	1.04	0.26
24–28	0.22	0.58	1.17	Immediately after the 28th hour the top water was poured out of the funnels and hot water added.	0.69	0.17
28–32	1.4	0.98	12.72		5.07	1.27
32–36	0.49	0.89	1.22	Artificial light	0.9	0.22
36–42	0.11	0.27	0.42	Dark	0.28	0.05
42–48	0.01	0.36	0.1	Natural and artificial light	0.18	0.03
48–54	0.06	1.07	0.26		0.54	0.09
54–60	3.75	0.85	0.37	Immediately after the 54th hour the top water was poured out of the funnels and hot water added.	1.44	0.24
60–72	0.65	0.57	0.37		0.51	0.042
72–84	1.53	0.89	1.1	A similar water change was made after the 72nd hour.	1.1	0.09
84–96	0.21	0.4	0.96	Dark for some hours	0.54	0.045
96–108	0.17	0.18	0.19	Natural and artificial light	0.18	0.015
108–120	0.17	0.13	0.26		0.19	0.016
120–132	0.83	0.27	0.64	Water changed immediately after 120th hour.	0.54	0.045
132–144	0.08	0.18	0.42	Dark for some hours.	0.24	0.02
144–156	0.42	0.27	0.48	Natural and artificial light	0.38	0.032
156–168	0.08	0.13	0.42		0.22	0.018
168–192	0.14	0.05	0.53	Water changed at 174th and 178th hours.	0.24	0.01
192–216	0.17	0.18	0.42	Cultures broken up and rebagged to release any imprisoned larvae at 193rd hour. Hot water added at 195th hour.	0.26	0.011
216–240	0.25	0.4	0.42	Water changed at 216th and 227th hours.	0.37	0.015
Total number of larvae.	35,900	56,000	47,100			

To determine the rate at which the larvae were extracted from cultures, three "control" cultures which were kept in the constant temperature room for 18 days, were drawn off at frequent intervals—hourly for the first six hours, every two hours for the next ten hours, every four hours for the following 20 hours, every six hours for the next 24 hours, then every 12 hours until the cultures had been in the funnels for a week. During this first week, except for the frequent drawing off of fluids, the cultures were treated in the usual manner, *i.e.*, the top waters were changed at the usual intervals, the lights were on in the room in which the funnels were kept for the usual periods, etc. During the three days after the end of the week everything was done to ensure that the larvae were extracted; hot water was frequently added, the cultures were broken up and rebagged with the centre on the outside, and lights were continually on. The larvae were counted every 24 hours.

The cultures were extracted in a nine-inch, an eight-inch and a six-inch funnel: they were first put in the funnels at ten o'clock in the morning.

Table IV shows the rate at which the larvae were extracted.

Where the larvae were numerous, the percentage, to two places of decimals, gives a false impression of accuracy, but two places of decimals are necessary when only a few were extracted.

The tests on the accuracy of the technique that has been employed, show that while large variations in the number of larvae isolated from controls containing numerous larvae have little importance, the utmost significance can be put on negative cultures.

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